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(54) Title: ASEXUAL INDUCTION OF HERITABLE MALE STERILITY AND APOMIXIS IN PLANTS

(57) Abstract

The present invention relates to methods for asexual induction of heritable male sterility and apomixis in plants. The invention is directed to factors derivable from certain plants which, when applied to certain recipient plants, induce heritable male sterility in the recipient. Such asexually transmissible male sterility factors, termed AMS/vectors, are present in extracts of certain male sterile alfalfa plants, where they are associated with a unique 1 x 10^6 (approx.) dalton molecular weight nucleic acid and a 40-110 nanometer particle. The asexually generated male-sterile plants derived by AMS/vector treatment can be used to produce new and valuable hybrids of alfalfa, corn, soybean, sorghum, sunflower, millet, tomato, and other plants.

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ASEXUAL INDUCTION OF HERITABLE MALE STERILITY AND APOMIXIS IN PLANTS

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1. FIELD OF THE INVENTION

This invention relates to asexual induction of heritable male sterility in plants. This phenomenon of induction and inheritance is hereinafter referred to as "asexual male sterility" or "AMS". The invention also relates to a method for induction of an apomictic-like phenomenon in plants, a phenomenon which may be associated with, but which is distinct from, male sterility. More particularly, this invention relates to factors derivable from certain plants which when applied to certain recipient plants induce heritable male sterility and/or apomixis in the recipient. These factors are hereinafter referred to as "AMS/vectors". The invention further relates to the use of such AMS/vectors in a rapid, asexual method for generating genetically diverse male sterile plants. Such plants can be used to produce new hybrids of importance in agronomy, horticulture, pomology and forestry.

2. BACKGROUND OF THE INVENTION

Monoecious plants are those in which male (staminate) and female (pistillate) organs are borne separately on the same individual plant. The male and female organs may be located in separate flowers, as in corn plants, or they may be in close physical juxtaposition, as in soybean plants. Monoecious plants occur widely in nature and are well represented among cultivated species, including important agricultural crops, horticultural varieties, as well as lumber, fruit and nut-bearing trees. Because monoecious plants have both male and female sex organs, they are capable of self-fertilization, i.e., pollen from the male organ can pollinate the female organ, giving rise to seed. Even in those monoecious plants which normally reproduce by cross-fertilization such as corn, where male and female

organs are located apart from each other on a plant, self-fertilization is possible.

While monoecy may be advantageous in nature, it can represent a problem in cultivar production. Indeed, it is frequently desirable that a cultivated monoecious plant be male-sterile so that it is incapable of self-fertilization. Situations in which male sterility is advantageous include the production of parthenocarpic fruits; the non-seed-setting of ornamentals thus giving long retention of flowers; and the production of doubleness in flowers where male sterility results in the transformation of anthers into petals. However, the most important instance by far in which male sterility is used advantageously as a breeding tool is in the production of hybrids, particularly F_1 (first filial generation) hybrids.

Hybridization is the cross-fertilization of one genetically unique plant by another. Its main virtues are to increase the genetic variation of plants and their progeny, to keep the population stable and to increase plant vigor. The increased plant vigor resulting from hybridization is referred to in the art as heterosis. In general, the greatest heterosis is observed when the least related genotypes are crossed together, e.g., crosses between unrelated cultivars tend to produce better hybrids than crosses between related cultivars because of the greater genotypic diferences.

Technically, an F_1 hybrid is the result of a cross between any two genetically distinct parent plants, regardless of their state of homozygosity. In the generally accepted connotation of the art, however, an F_1 hybrid is the product of a cross between two homozygous (but genetically distinct) parents or lines, and all F_1 plants resemble one another exactly. The recognized advantages of F_1 hybrids are: a) greater vigor expressed

as, inter alia, improved yield, flower or seed production, earli r germination, dis ase resistance, insect r sistance and other manif stations of h t rosis; b) greater adaptability to varying environmental conditions because the majority of genes are present in the heterozygous state; c) the expression of advantageous characters when these are controlled by dominant alleles; and d) control by the breeder over the resulting hybrid product.

Because many of the plants that breeders want to make hybrids from are monoecious, i.e., capable of undergoing self-fertilization as well as cross-fertilization, the desired hybridization is difficult to achieve on a reliable basis particularly on a commercial level. Thus, the goal in any hybridization program involving monoecious plants is to control or facilitate cross-fertilization by minimizing, or preferably eliminating, self-fertilization. One way to attain this goal is to use a male-sterile plant as one of the parents in the breeding scheme.

In the past, male sterility of parental lines has been achieved in a variety of ways, all fraught with a variety of drawbacks. For example, monoecious plants may be made male-sterile by physically (either manually or mechanically) removing the male flowers, organs or pollen-bearing anthers from the plant. This approach can be labor-intensive and, given human and machine error, not particularly fail-safe. Physical emasculation in the field is weather-dependent and can result in loss of tissue and yield. Alternatively, monoecious plants may be treated with chemicals such as gametocides, which destroy the ability of the plant to yield viable pollen, or chemical hybridizing agents, which do not affect pollen viability but prevent pollen from causing self-fertilization. However, this approach can be costly and/or lead to deleterious environmental effects.

The most frequently encountered approach to male sterility in monoecious plants is through biological means which result in an inability of the plant to produce viable pollen. One type of biological male sterility is known in the art as genetic male sterility (Allard, Principles of Plant Breeding, John Wiley & Sons, New York, 1960, p. 245; Watts, Flower & Vegetable Plant Breeding, Grower Books, London, 1980, p. 42). Briefly, in some plants, the male-sterile or male-fertile state is dependent on a single gene. Plants homozygous for the recessive allele are male-sterile and can be used as parental lines for hybrid production. The homozygous male-sterile line is maintained by crossing it with a known heterozygote (for the sterility/fertility alleles) which yields 50% homozygous male-sterile progeny and 50% heterozygous male-fertile progeny. Care must be taken to use only the homozygous male-sterile progeny as maternal parent for the subsequent hybridizations. Care must also be taken not to allow the heterozygotes to intercross with one another as that will result in homozygous male-fertiles, upsetting the system. Overall the approach is not dependable.

Another type of biological male sterility is known in the art as cytoplasmic male sterility, or CMS, and is dependent on cytoplasmic factors. See Allard, supra, at pp. 245-246. Plants carrying particular types of cytoplasm are male-sterile and can be used as parental lines to make F_1 hybrids. These F_1 hybrids are all male-sterile since their cytoplasm is derived entirely from the female gamete (from the male-sterile parent). In other words, the CMS trait is maternally inherited.

Many maize cytoplasms which can confer the trait of male sterility belong to the S group, which has been shown to contain three plasmid-like DNAs in the mitochondria (Sisco, P.H., et al., 1984, Plant Science

Letters 34:127-134; Pring, D.R., et al., 1977, Proc. Natl. Acad. Sci. U.S.A. 74:2904; Kemble, R.J., et al., 1980, Genetics 95:451; Koncz, C., et al., 1981, Mol. Gen. Genet. 183:449). S cytoplasms do not show stable male sterility (Laughnan, J.R. and Gabay-Laughnan, 1983, Ann. Rev. Genet. 17:27-48) and in some genetic backgrounds have a high rate of reversion to male fertility.

Yet another type of biological male sterility is sometimes referred to as cytoplasmic-genetic male sterility (see Allard, supra, at pp. 246-247). It differs from cytoplasmic male sterility only in that the offspring of male-sterile (maternal) plants are not necessarily male-sterile but can be made male-fertile if plants of a certain genetic make-up are used as the paternal parent. These paternal parents that produce male-fertile F_1 progeny carry genes with the power to restore the pollen-producing ability of plants with male-sterile cytoplasm. These genes are known as restorer genes and the plants that carry them, restorers. Such cytoplasmic-genetic male sterility has been put to use in, e.g., onion breeding (See, Jones and Davis, 1944, U.S.D.A. Technical Bulletin 874:1-28).

Creation of a new male-sterile parent for production of hybrids by means of cytoplasmic male sterility or cytoplasmic-genetic male sterility requires laborious and time-consuming sexual transmission through backcrossing. The scheme for sexual transmission of cytoplasmic male sterility, which may be more accurately described as the transfer of a genotype or nuclear component to a male-sterility-producing cytoplasm, is set forth in Allard, supra, at pp. 246-247.

The seed industry has long used sexually transmitted cytoplasmic male sterility for pollination control in the production of hybrid seed products. However, sexually transmitted cytoplasmic male sterility

is carried in very few varieties of any one species, and, as mentioned previously, transmission is a time-consuming and expensive process requiring numerous generations of breeding to arrive at a new male sterile parental line.

In addition to the time, effort and expense of multiple breeding generations, the use of sexually transferred cytoplasmic male sterility has led to a very narrow cytoplasmic base as the cytoplasms are not genetically altered by conventional pollination. This has had deleterious consequences. For example, in 1970, more than 85% of the corn grown in the United States carried the T-strain of CMS cytoplasm due to the success achieved using CMS lines in the production of hybrid corn. However, in that same year, an epiphytotic of southern corn leaf blight destroyed a large percentage of the corn crop; this disease is caused by race T of Helminthosporium maydis, an ascomycete which is particularly virulent on plants with CMS-T cytoplasm.

Because of the inherent drawbacks of breeding programs that rely on sexual transmission of cytoplasmic male sterility, workers in the art have sought asexual means for transmitting cytoplasmic factors responsible for male sterility. One asexual means is grafting. Male sterility has been shown to be graft transmissible (although it is not expressed until the F_1 generation) in such plants as petunias (Frankel, 1956, Science 124:684-685; Edwardson and Corbett, 1967, Proc. Natl. Acad. Sci. U.S.A. 47:390-396; Frankel, 1962, Genetics 47:641-646) and alfalfa (Thompson and Axtell, 1978, J. Hered. 69:159-164). The problem with this approach is that transmission of male sterility is achieved only at low frequency.

Cytoplasmic male sterility factors have also been asexually transmitted by means of somatic fusions. Protoplasts from different plants are fused in culture to form hybrids, sometimes called 'cybrids'. Such a

technique has been used by B lliard and Pelletier in tobacco (1980, Eur. J. C ll Biol. 22(1):605). The major drawbacks of somatic fusion as an asexual means of cytoplasmic male sterility transmission are very low regeneration frequencies and the need for appropriate screens or markers for selecting the fused hybrids in vitro. Another asexual technique that has been used for the transfer of cytoplasmic male sterility is transmission through an intermediate host such as dodder (Cuscuta sp.). Such an intermediate host is known in the art as a dodder bridge. The major drawback of this approach is that dodder itself is considered a noxious parasite, both a weed and a disease, and therefore is not a likely candidate for large-scale field use.

Grill and Garger (1981, Proc. Natl. Acad. Sci. U.S.A. 78(11):7043-7046) have used the dodder bridge with Vicia faba (fava bean plant). They identified and characterized a high molecular weight double-stranded RNA (dsRNA) associated with cytoplasmic male sterility in Vicia faba. The dsRNA is apparently located in spherical bodies, 70 nanometers in diameter, located in the cytoplasm of the plant, much like a virus. The dsRNA was transmitted to a fertile line of V. faba by first growing dodder on the CMS V. faba and then contacting this dodder with a male fertile plant. After removing the dodder from the recipient, its flowering was observed. Sixty percent of previously male-fertile plants so treated had become male-sterile and now contained the dsRNA characteristic of the original male-sterile plants.

Though successful, grafting, protoplast fusion and use of dodder bridges as means of asexual transmission of cytoplasmic male sterility are laborious and not well-suited for large-scale operations.

Cytoplasmic sterility has also been induced by mutagenesis, by exposure to ethidium bromide for pearl

millet (Burton, G.W. and Hanna, W.W., 1976, Crop Science 16:731-732), and by treatment with EMS for rice (Mallick, E.H., 1980, Genet. Agr. 34:207-213).

Two maternally transmitted nucleic acid species comprising double-stranded RNAs of molecular weights 1.9 X 10^6 and 0.5 X 10^6, have been shown to be associated with the mitochondria in a male-sterile cytoplasm of maize, termed LBN cytoplasm (Sisco, P.H., et al., 1984, Plant Science Letters 34:127-134; U.S. Patent No. 4,569,152 by Gracen et al., filed April 26, 1984). Plasmid-like DNAs have also been detected in the mitochondria of source IS1112C male-sterile sorghum cytoplasm (Pring, D.R., et al., 1982, Mol. Gen. Genet. 186:180-184).

3. SUMMARY OF THE INVENTION

It is an object of the present invention to provide a means for inducing heritable male sterility in plants that overcomes the drawbacks of prior art methods for achieving male sterility. It is thus an object of the present invention to provide a rapid asexual method for inducing male sterility that avoids the laborious, expensive and time-consuming aspects of physical emasculation, chemical treatments, backcrossing in sexual transmission, grafting, protoplast fusion and intermediate host bridging.

It is a further object of the invention to provide an asexual means for inducing heritable male sterility in plants that is adaptable to large scale generation of new lines useful in and of themselves and new parental lines for the commercial production of new and useful hybrids exhibiting heterosis. In this latter regard, it is an object of the invention to provide a means for asexually inducing male sterility that is subsequently inherited by progeny of the male sterile line

so induced to increase the number of male-sterile parents for commercial scale hybrid production.

It is also an object of the invention to increase genetic diversity among male sterile parental lines used in hybridizations by inducing male sterility in plants which heretofore were available only as male fertile parental lines. A further object of the invention is to so provide hybrids of agronomic, horticultural, forestry and pomological importance with high yields, disease resistance, pest resistance and/or resistance to adverse environmental conditions.

It is a further object of the invention to provide a versatile asexual means for transferring heritable male sterility between plants not only of different species but of different genera, as well as between dicots and monocots.

It is a further object of the invention to provide a means for inducing apomixis in plants which permits the perpetuation of agromically desirable hybrid lines in a more convenient and efficient manner than has previously been possible with a large number of plant species. Establishment of apomixis allows the development of seed, identical in genetic composition with the female parent, without the necessity for gametic fusion. In this regard, it is an object of the invention to provide a means for inducing apomitic reproduction, which characteristic is inherited by subsequent progeny, thereby avoiding the need for repeated crossings of selected parental lines in order to continuously produce hybrid seed.

These and other objects can be achieved by the materials and methods provided herein. The invention is directed to asexually transmissible male sterility and apomixis factors, AMS/vectors, present in extracts from certain male sterile alfalfa plants. Characteristically

associated with such extracts, and treated sterile plants, are (1) an unique isolatable nucleic acid with a molecular weight of about 1 x 10^6 daltons; and (2) particles, about 40-110 nanometers in diameter, consisting of a dense core surrounded by a bilayer membrane, as observed microscopically. The invention is further directed to such extracts and their use in asexually inducing male sterility and/or apomixis in recipient plants. More specifically, the extracts from alfalfa plants displaying the AMS trait, when applied, e.g., by spraying, to susceptible recipient plants, induce or impart male sterility in the recipient. These extracts have also been demonstrated to induce or impart an apomictic form of reproduction in plants so treated. Remarkably, the AMS/vector extracts are effective in inducing male sterility or apomixis across species and genera as well as between dicots and monocots. The invention further provides improved methods for the production of F_1 hybrid plants wherein the improvement comprises using asexually AMS/vector-induced male sterile plants as the maternal parent in F_1 crosses.

The invention also provides a method of producing hybrid seed in which the improvement comprises crossing two parent lines, one of which has been treated with AMS/vector, to produce F_1 hybrid progeny, using the F_1 progeny to produce F_2 progeny, identifying those F_2 plants which are identical in phenotype to the F_1 and which set seed, propagating such plants, and collecting hybrid seed therefrom. The invention further provides hybrid seed capable of producing apomictic plants, as well as the apomictic plants derived therefrom.

The invention also contemplates the use of the 1 X 10^6 (approx.) dalton nucleic acid and/or 40-110 nm particle, uniquely associated with extracts containing the

AMS/vector, as a transmissible plant delivery or expression vector system.

3.1. DEFINITIONS

The following abbreviations are used herein and shall have the meanings indicated:

AMS = asexual male sterility
CMS = cytoplasmic male sterility
DNase = deoxyribonuclease
RNase = ribonuclease
kb = kilobase pair
OBS = observation; an experimental treatment group
REP = replication
TRT = treatment

4. BRIEF DESCRIPTION OF THE FIGURES

Fig. 1A is a photograph of an ethidium bromide-stained agarose gel in which nucleic acids extracted from alfalfa AMS/vector source 1.29 (U.S.D.A. PI No. 223386) (lane 1), from an untreated fertile alfalfa maintainer (variety Arc) (lane 3), and from an untreated fertile alfalfa non-maintainer (lane 4) were run. A single band at approximately 3.5 kb associated with the AMS/vector source is seen in lane 1, but not in lanes 3 or 4. Lane 2 in Fig. 1A is a HindIII digest of bacteriophage lambda DNA, with molecular weights of (from top to botom) 23.6 kb, 9.6 kb, 6.6 kb, 4.3 kb, 2.2 kb, and 1.9 kb.

Fig. 1B is a photograph of an ethidium bromide-stained agarose gel in which nucleic acids extracted from fertile alfalfa (variety Arc) (lane 1) and from alfalfa (variety Arc) converted to male sterility by treatment with AMS/vector source 1.29 (U.S.D.A. PI No. 223386) (lane 3) were run. A single band at approximately 3.5 kb

associated with the AMS trait is seen in Figure 1B, lane 3, but not in lan 1. Lane 2 in Fig. 1B is a HindIII digest of bacteriophage lambda DNA, as described for Fig. 1A..

Fig. 1C is a photograph of an ethidium bromide-stained agarose gel in which nucleic acids extracted from corn (variety B73) converted to male sterility by treatment with AMS/vector source 1.26 (U.S.D.A. PI No. 221469) (lane 1) and fertile corn (variety B73) (lane 2) were run. A single band at approximately 3.5 kb associated with the AMS trait is seen in Fig. 1C, lane 1, but not in lane 2. Lane 3 is a HindIII digest of bacteriophage lambda DNA, as described for Fig. 1A.

Fig. 1D is a photograph of an ethidium bromide-stained agarose gel in which nucleic acids extracted from soybean (variety Williams 82) converted to male sterility by treatment with AMS/vector source 1.36 (U.S.D.A. PI No. 243223) (lane 1) and fertile soybean (variety Williams 82) (lane 2) were run. A single band at approximately 3.5 kb associated with the AMS trait is seen in Fig. 1D, lane 1, but not in lane 2. Lane 3 is a HindIII digest of bacteriophage lambda DNA, as described for Fig. 1A.

Fig. 1E depicts the results of the experiment described infra in Section 6.2, demonstrating the DNA nature of the approximately 3.5 kb nucleic acid associated with extracts containing the AMS/vector. Nucleic acids extracted from alfalfa were subjected to treatment with either DNase (lanes 1-7) or RNase (lanes 8-14) before agarose gel electrophoresis and ethidium bromide staining. Fig. 1E is a photograph of the ethidium bromide staining pattern. Lane 1, HindIII digest of bacteriophage lambda DNA (as described for Fig. 1A); lane 2, restorer alfalfa line Indiana Synthetic (C); lane 3, AMS/vector source 1.26 (U.S.D.A. PI No. 221469); lane 4, AMS/vector source 1.36 (U.S.D.A. PI No. 243223); lane 5, alfalfa maintainer

(variety Arc); lane 6, AMS/vector source 1.29 (U.S.D.A. PI No. 223386); lane 7, AMS/vector source 1.7 (U.S.D.A. PI No. 173733); lane 8, HindIII digest of bacteriophage lambda DNA (as described for Fig. 1A); lane 9, restorer alfalfa line Indiana Synthetic (C); lane 10, AMS/vector source 1.26 (U.S.D.A. PI No. 221469); lane 11, AMS/vector source 1.36 (U.S.D.A. PI No. 243223); lane 12, alfalfa maintainer (variety Arc); lane 13, AMS/vector source 1.29 (U.S.D.A. PI No. 223386); lane 14, AMS/vector source 1.7 (U.S.D.A. PI No. 173733). The approximately 3.5 kb band (indicated by the arrow) present in AMS/vector sources remains after RNase treatment, but is absent after DNase treatment.

Fig. 2A is an electron micrograph of the 40-110 nanometer particles present in a crude extract of a male-sterile alfalfa plant, U.S.D.A. PI No. 223386. Magnification: 20,000 X.

Fig. 2B is an electron micrograph of the 40-110 nanometer particles observed in an ovule of a male-sterile alfalfa plant, U.S.D.A. PI No. 221469. Magnification: 10,000 X.

Fig. 2C is an electron micrograph of a thin section of a seed from a cross between an alfalfa maintainer plant and a formerly fertile alfalfa plant that was converted to male sterility by treatment with extracts of an alfalfa AMS/vector source, U.S.D.A. PI No. 223386. The white inclusion bodies exhibiting dark spots may contain the approximately 3.5 kb nucleic acid associated with extracts of the AMS/vector. Magnification: 20,000 X.

Fig. 3 (3A, 3B, 3C, 3D) contains photographs of representative microscopic fields depicting the pollen present in anthers from tassels containing dehisced pollen for varieties 1-4 of Zea mays L. corn plant, from the field test described in Section 6.9, infra.

Fig. 4 contains a photograph of a representative microscopic field depicting the anthers from tassels that showed no dehisced pollen, for variety 2 of Zea mays L. corn plant, from the field test described in Section 6.9, infra.

Fig. 5A contains a photograph of a representative microscopic field depicting the release of pollen from anthers of variety 1 as shown in Fig. 4, after the application of pressure.

Fig. 5B contains a photograph of a representative microscopic field depicting the release of pollen from anthers of variety 2 as shown in Fig. 4, after the application of pressure.

Fig. 6 contains a photograph of a representative microscopic field depicting the absence of observable sporogenous tissue in anthers with no dehisced pollen, after the application of pressure, in variety 4 of Zea mays L. corn plant, from the field test described in Section 6.9, infra.

Fig. 7 is a photograph of a representative microscopic field depicting anthers with abundant pollen grains of uniform size and shape, in a treated soybean plant (Glycine max var. Williams 82) from the growth room test described in Section 6.10, infra.

Fig. 8 is a photograph of a representative microscopic field depicting the red staining with acetocarmine of anthers as shown in Fig. 7.

Fig. 9 is a photograph of a representative microscopic field depicting the characteristic mass of pollen grains from anthers, as shown in Fig. 7, attached to stigma.

Fig. 10 is a photograph of a representative microscopic field depicting anthers containing a mix of non-stainable, abnormally shaped pollen grains and normal pollen, from a treated soybean plant (Glycine max var.

Williams 82) from the growth room test described in Section 6.10, infra.

Fig. 11 is a photograph of a representative microscopic field depicting the irregular shape, lack of staining with acetocarmine, and high degree of vacuolation of anthers as shown in Fig. 10.

Fig. 12 is a photograph of a representative microscopic field depicting anthers which lack any pollen grains, from a treated soybean plant (Glycine max var. Williams 82) from the growth room test described in Section 6.10, infra.

Fig. 13 is a photograph of a representative microscopic field depicting the absence of any observable pollen grains in anthers as shown in Fig. 12, after the application of pressure.

Fig. 14 is a photograph of representative sterile tassels of "inbred 1" Zea mays L. corn plant, from the experiment described in Section 6.11, infra. There is no visible dehiscence of anthers. Such tassels did not shed pollen, and were rated sterile.

Fig. 15 is a photograph of representative sterile tassels of "inbred 2" Zea mays L. corn plant, from the experiment described in Section 6.11, infra. There is no visible dehiscence of anthers. Such tassels did not shed pollen, and were rated sterile.

Fig. 16 contains photographs of representative tassels of inbred Zea mays L. corn plants, from the experiment described in Section 6.11, infra. Part A shows a fertile tassel of inbred 1, exhibiting dehisced anthers. (The mass of pollen on the blue paper is apparent.) Part B shows a tassel of inbred 1, rated sterile. Part C shows a tassel of inbred 2, rated fertile. Part D shows a tassel of inbred 2, rated sterile.

Fig. 17 contains photographs of representative tassels of inbred Zea mays L. corn plants, from the

experiment described in Section 6.11, infra. Part A shows a tassel of inbred 4, rated fertile. Part B shows a tassel of inbred 4, rated sterile. Part C shows a tassel of inbred 2 rated fertile, but showing only one dehisced anther. Part D shows a tassel of inbred 4 rated fertile, showing up to ten dehisced anthers.

Fig. 18 contains photographs of representative microscopic fields depicting the results of acetocarmine staining of anthers from tassels of inbred Zea mays L. corn plants, from the experiment described in Section 6.11, infra. Part A shows normal, round, and stainable pollen of inbred 1 from tassels rated fertile. Part B shows pollen from tassels of inbred 2, rated fertile. Part C shows pollen from tassels of inbred 3, rated fertile. Part D shows pollen of tassels of inbred 4, rated fertile.

Fig. 19 contains photographs of representative microscopic fields depicting the results of acetocarmine staining of anthers from tassels of inbred Zea mays L. corn plants, from the experiment described in Section 6.11, infra. Part A shows a fully dehisced anther from tassels of inbred 1, rated fertile. The anther wall and a single pollen grain are apparent. Part B shows a fully dehisced anther from tassels of inbred 2, rated fertile. Part C shows a fully dehisced anther from tassels of inbred 3, rated fertile. Part D shows a fully dehisced anther from tassels of inbred 4, rated fertile.

Fig. 20 contains photographs of representative microscopic fields depicting the results of acetocarmine staining of anthers from tassels of inbred Zea mays L. corn plants, from the experiment described in Section 6.11, infra. Part A shows abnormal pollen in the anthers from a tassel of inbred 1, rated sterile. Part B shows abnormal pollen in the anthers from a tassel of inbred 2, rated sterile. Part C shows abnormal pollen in the

anthers from a tassel of inbred 3, rated sterile. Part D shows no detectable pollen in the anther from a tassel of inbred 4, rated sterile.

Fig. 21 contains photographs of representative microscopic fields depicting the results of acetocarmine staining of anthers from tassels of inbred Zea mays L. corn plants, from the experiment described in Section 6.11, infra. Part A shows anthers from a tassel of inbred 1, rated sterile, crushed to reveal abnormal, irregularly shaped, non-stainable pollen. Part B shows anthers from a tassel of inbred 2, rated sterile, crushed to reveal abnormal, irregularly shaped, non-stainable pollen. Part C shows anthers from a tassel of inbred 3, rated sterile, crushed to reveal abnormal pollen. Part D shows anthers from a tassel of inbred 4, rated sterile, revealing no pollen after crushing.

Fig. 22 contains photographs of representative microscopic fields depicting the results of acetocarmine staining of anthers from tassels of inbred Zea mays L. corn plants, from the experiment described in Section 6.11, infra. Part A shows anthers from tassels of inbred 1, rated sterile, exhibiting a few stainable, normal looking pollen, which were uncommon. Part B shows predominantly abnormal and a few normal looking pollen in an undehisced anther from a tassel of inbred 2, rated fertile. Parts C and D show anthers as described in Part B, revealing bulged portions which lodged predominantly normal looking pollen.

5. DETAILED DESCRIPTION OF THE INVENTION

5.1. SOURCES OF AMS/VECTOR

Nondomestic alfalfa plants (genus Medicago) of Middle Eastern origin can serve as sources (donors) of AMS/vectors. Plants obtained from the Seed Increase

Collection, U.S.D.A., Reno, Nevada (1979-1984) were screened for insect resistance and reduced seed set. Out of approximately seventeen thousand plants, five were selected as bearing the AMS/vector trait, i.e., all possessed an extractable factor which when applied to susceptible recipients imparted male sterility. All the AMS/vector-bearing plants were characterized as being male sterile, tetraploid, purple-flowered perennials. Extracts of these plants characteristically contained particles about 40-110 nanometers in diameter and an isolatable nucleic acid with a molecular weight of about 1.1×10^6 daltons (see Section 6.2.). The specific plants which can serve as a source of the AMS/vector in a particular embodiment as described herein had the following Plant Introduction numbers (PI Nos.) when obtained from the Seed Increase Collection: PI No. 172429, PI No. 173733, PI No. 221469, PI No. 223386, and PI No. 243223. Seeds from plants resulting from crosses between each of the five sources and Arc-derived maintainer plants (Medicago sativa var. Arc developed at the U.S.D.A. Labs, Beltsville, MD) can be used to generate plants which can also serve as sources of AMS/vectors.

Other sources of AMS/vectors may exist. They may be determined empirically by following the methods of Sections 5.2. and 5.3.

5.2. PREPARATION AND APPLICATION OF AMS/VECTOR EXTRACTS

Preparations containing the AMS/vector may be prepared by a simple extraction procedure. Donor alfalfa plants are harvested when they have fully developed crowns, usually at one-tenth bloom or a week before. Leaves and stems are used fresh or stored frozen for future use. The plant material is suspended in any suitable non-lethal buffer such as potassium phosphate

buffer (e.g., 0.067 M KH_2PO_4 at pH 6.9). Typically, for every five to seven ml of buffer, about one gram of plant tissue is suspended therein. Other ingredients may be added to the extract such as abradors (e.g., diatomaceous earth such as Celite) or absorption enhancers (e.g., dimethylsulfoxide or DMSO). The plant material is macerated by any suitable means, e.g., blending in a high speed blender to form a homogenate. Residual plant debris is removed by filtration, decantation or other suitable means. The extraction procedure need not be performed under sterile conditions and the resulting filtrate or extract need not be stored in sterile containers. The extract may be kept refrigerated for periods up to approximately three hours before use. Otherwise, it may be stored frozen, e.g., in liquid nitrogen, until use.

AMS/vector extracts thus prepared are sprayed on recipient plants using standard field equipment. In general, only one application of the extract is necessary. In a particular embodiment, about 5 to 25 milliliters (ml) can be applied per plant. The extract is sprayed onto the leaves of the recipient. The inclusion of an abrador (e.g., Celite) is preferred.

Recipient plants are to be sprayed at a time when they have foliage, but prior to flowering and seed set. For example, soybean plant recipients may be sprayed at least about two weeks after germination; earlier application does not result, or results poorly, in induction of male sterility. Corn plant recipients may be sprayed when the fifth leaf is exposed, at the beginning of the grand growth stage, approximately three weeks after germination. In the case of alfalfa, recipients are cut back about two centimeters above the crown; within a two-week period of time, the alfalfa recipients may have extracts applied to them.

Other methods of application are possible including, but not limited to, tissue culture (suspension of plant tissue in media containing AMS/vectors), electroporation of the AMS/vectors into protoplasts (e.g., for vegetable crops) and injection (e.g., for trees).

5.3. PLANTS INDUCIBLE TO MALE STERILITY BY AMS/VECTORS

All plants are potentially inducible to male sterility by the AMS/vector if genetically predisposed to inducibility. This includes monoecious plants and even dioecious plants (i.e., plants in which male and female organs occur on different individuals) where, as a result of inducing male sterility, a male plant is transformed into a female plant. Without desiring to be bound by the following proposed theory, it is hypothesized that in inducible recipients, all chromosomes carry the recessive allele for inducibility of male sterility mediated by the AMS/vector. For example, if the recessive allele for inducibility of male sterility is denoted "r", to be an inducible recipient, a tetraploid (e.g., alfalfa) would have to be in the "rrrr" state while a diploid (e.g., soy or corn) would have to be in the "rr" state within the nucleus of the cells of the plant.

Plants which are of greatest interest are those of agronomic and horticultural importance, including, but not limited to, grain crops, forage crops, seed propagated fruits, seed propagated ornamentals and industrial species. Representative monoecious plants which may be used as recipients of the AMS/vectors to create new male sterile plants are listed in Table I. The table is presented by way of illustration and is by no means exhaustive.

TABLE I.

MONOECIOUS PLANTS INDUCIBLE TO MALE STERILITY BY AMS/VECTORS

Grain Crops	Fruits
Cereals	Tomatoes
Corn	Peppers
Wheat	Watermelons
Barley	Apples
Sorghum	Oranges
Rye	Grapefruits
Oats	Lemons
Rice	Limes
Grain legumes	Forage Crops
Field beans	Alfalfa
Peas	Onions
Peanuts	Peppers
Lentils	Sugar Beets
	Turnips
Seed Propagated	Broccoli
Ornamentals	Cabbage
Petunias	Potatoes
Marigolds	
	Industrial Species
	Poplar Trees
	Maple Trees
Oilseeds	Cotton
Soybeans	Tobacco
Sunflower	Fibre Flax
Flax	Kelp
Mustard	
Safflower	
Rape	

Recipient plants inducible to male sterility by AMS/vectors may be identified by applying xtracts as described in Section 5.2. and visually rating the recipient plant with regard to pollen production and seed set. Those which do not produce pollen and/or seed are inducible recipients.

This invention contemplates the use of DNA probes to identify inducible recipients. The DNA of known inducible and non-inducible plants may be subjected to restriction endonuclease digestion. Fragments unique to the inducible plants may be identified and serve as a template from which to make DNA probes. These probes may then be used to screen, via hybridization methodologies, for other recipients (see Maniatis, T., et al., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). Alternatively, probes may be used to identify induced plants where unique nucleic acids are associated with plants exhibiting the AMS trait.

5.4. USE OF AMS/VECTOR-INDUCED MALE STERILE PLANTS TO PRODUCE HYBRIDS

The AMS/vector-induced male sterile plants may be used as the maternal parental line in hybridization schemes known in the art. Such male-sterile maternal lines may be maintained or expanded in number by crossing them with 'maintainers', i.e., the genetically identical, non-AMS/vector-treated plant.

The choice of paternal lines for crossing with the AMS/vector-induced male sterile maternal lines varies, depending on the intended use of the F_1 offspring. If the F_1 offspring plants are desirable in and of themselves as, e.g., forage crops or ornamentals, it is not necessary that the F_1 hybrids be male-fertile and hence capable of producing seed. Thus it is not necessary to choose a male

parental line that will result in the F_1 hybrids being male fertile.

However, if the F_1 offspring plants are desired to b s ed produc rs, a restorer may be used as the male parental line. Restorers are identified by performing the cross and observing the percent fertility of the F_1 progeny. Male parental plants, which when crossed with the male-sterile female parental plants yield fertile F_1 progeny, are considered restorers. By way of illustration for inbred corn lines, B73 is a known restorer of AMS/vector-induced sterile Mo17; Mo17 is a known restorer of AMS/vector-induced sterile B73; and H95 is a known restorer of AMS/vector-induced sterile A632. Generally, the more unrelated two inbred lines are, the more likely one will act as a restorer for the other and vice versa.

As an alternative to restorers, male fertile plants, which when crossed with male-sterile maternal plants yield F_1 progeny that are vegetatively propagated through seed, may be used as the paternal plant for production of seed-bearing F_1 hybrids.

Both the F_1 progeny and the male-sterile plants containing the AMS/vector can, in addition to other methods, be propagated vegetatively. The stem of the plant can be cut off at the base, placed in rooting medium and allowed to root, before being transplanted to soil. Tissue culture methods of propagation are also envisioned for use (for review, see Vasil, I., et al., 1979, in Advances in Genetics, Vol. 20, Caspari, E.W., ed., Academic Press, New York, pp. 127-216).

5.5. INDUCTION OF APOMIXIS

5.5.1. ASEXUAL REPRODUCTION IN HIGHER PLANTS

Although, as a rule, higher plants routinely reproduce sexually, i.e., by way of gametic fusion, there

are, among certain types of plants, episodes of various types of asexual reproduction. Some varieties may typically be reproduced asexually by artificial vegetative propagation. This technique is frequently used by plant breeders in plants with poor seed set; it may also be used to eliminate an undesired genetic variability which may result from seed propagation. Vegetative propagation may be achieved by roots, tubers, stolons, rhizomes, stem or leaf cuttings, or tissue culture; those plants obtained in this manner are, absent a mutation, genotypically and phenotypically identical to the parent plant. A number of well-known commercial crops are routinely produced in this manner. For example, stem sections are frequently used in the propagation of sugarcane, which only rarely produces flowers in non-tropical regions. Similarly, roots and tubers are employed in the production of root crops such as cassava, sweet potatoes, potatoes, and yams.

A very different type of asexual reproduction, which does involve setting of seed, is known as apomixis. In this form of reproduction, which occurs spontaneously, i.e., without human intervention, in hundreds of plant species. The sexual organs and related structures take part in reproduction, but the seeds which are formed are produced without union of gametes. In certain plant species, apomixis is the only form of reproduction, and these plants are known as obligate apomicts. Frequently, however, the apomictic plant will exhibit both gametic and apomictic reproduction, and these plants are referred to as facultative apomicts. In the latter group, the sexual and asexual processes may operate simultaneously in an individual plant.

In both obligate and facultative apomicts, there may be several mechanisms or combinations of mechanisms involved in the asexual process. There are four basic types of apomixis. In apogamy, the embryo develops from

two haploid nuclei other than the eggs; frequently it results from the fusion of two cells of the embryo sac, either synergids or antipodal cells. In apospory, the embryo sac develops directly from a somatic cell without reduction and formation of spores; the embryo develops from the diploid egg without fertilization. In diplospory, the embryo develops from the megaspore mother cell without reduction. Finally, in parthenogenesis, the embryo develops directly from an unfertilized egg and may or may not be haploid, depending on the regularity of meiosis which produces the egg. For purposes of the present discussion, the term apomixis will be used generically to apply to any or all of these phenomena, or any variation which produces the same end result. It is generally believed that apomixis is controlled genetically (Taliaferro, C.M., Southern Pasture Forage Crop Impr. Conf. Rep. 26:41-43, 1969) and it has been suggested that it may be controlled by a single gene (Harlan et al., Bot. Gaz. 125:41-46, 1964).

5.5.2. USE OF APOMIXIS IN BREEDING

When first discovered, apomixis was considered to be a complete barrier to plant breeding. Hybridization between obligate apomicts is virtually impossible except in the rarest of circumstances. In most types of apomixis, the embryo has the same genetic constitution as the maternal plant, and is a true clone. Thus, the possibility of introducing variation into an apomictic line for the purpose of developing new varieties or hybrids, would appear to be severely limited. In fact, early workers generally considered apomixis as an evolutionary "blind alley" (Darlington, The Evolution of Genetic Systems, p. 149 University Press, Cambridge, 1939) because of the potential for reproductive isolation.

In recent years, however, it has become apparent to plant breeders that the phenomenon may have valuable applications in breeding. If apomixis could be controlled completely, a means is provided whereby a producer would have available a system which provides the consistency and reliability of breeding through vegetative organs, but with the convenience of seed propagation. Further, the breeder attains the advantage of being able to experiment with various parental pairings to isolate superior hybrid combinations, and to simultaneously "fix" the heterosis by obtaining a true breeding F_1. This technique could prove particularly valuable in those crops in which hybrid seed production in commercial quantities has been hampered by low seed set due to inadequate pollination. Such important crops include, for example, wheat, soybean and cotton.

5.5.3. AMS/VECTOR INDUCTION OF APOMIXIS

In addition to the observed effect on male sterility which can be obtained by treatment of plants, it has also been unexpectedly discovered that AMS/vector has the ability to induce apomixis in treated plants. In the process of study of the pattern of inheritance of male sterility in AMS/vector-treated plants, certain initial observations in the inheritance of other phenotypic characteristics indicated that some treated plants were not exhibiting a pattern which would be expected from normal hybrid production between sexually reproducing parents.

For example, crosses were performed between phenotypically distinct soybean parent lines, one parent of which had been treated with AMS/vector, and were shown to be male sterile. Soybean is not known to be naturally apomictic. The chosen male-sterile plants, used as female parents, produced a white flower and a green hypocotyl;

the male fertile plant, used as male parent, produced a purple flower and purple hypocotyl. Each of these is controlled by a single gene. The F_1 produced by this cross all exhibited, as expect d, the purple flower, purple hypocotyl phenotype; among these plants were a substantial percentage of male steriles, of which a small percentage set seed. The F_1 plants were then selfed to produce an F_2 generation. If normal patterns of sexual propagation and inheritance were occurring, it would be expected that the resulting F_2 generation should segregate for flower and hypocotyl color. Surprisingly, however, there was virtually no segregation for flower and hypocotyl color, and again, a significant number of the plants in the F_2 were male sterile which set seed. This pattern is not only contrary to what would be expected in normal sexual reproduction, but is consistent with a pattern which characterizes apomictic seed production, namely: (1) an absence of the expected segregation among progeny of an F_1 hybrid cross; and (2) the occurrence of male sterile progeny which still set seed. This pattern observed was repeated in subsequent F_3 and F_4 generations, although some breakdown was observed in the F_5 generation. Nonetheless, it appears clear that apomixis, or an apomixis-like phenomenon, is inducible by application of AMS/vector to a susceptible plant. A similar pattern has also been observed in preliminary trials with wheat and corn.

The treatment of plants to induce apomixis can be achieved in much the same manner as is the induction of male sterility. A usual method of application is spraying the subject plants at a time prior to flowering, but at a time when the plant is sufficiently mature to have developed foliage. Alternately, it may be desirable to spray shortly after flower initiation, in order to attempt to directly affect the developing seed. The manipulations

necessary to determine the optimum pattern of application for a given type of plant is well within the skill of the experienced plant breeder.

Following application of AMS/vector, the seeds resulting from the cross are planted and grown to maturity, this group constituting the F_1 hybrid generation. All members of the F_1 should be identical in phenotype. Among these will usually be a number of male sterile plants resulting from the treatment. The F_1 plants are allowed to self. The seed is collected and planted, and the phenotypes of the resulting F_2 generation observed. If no induction of apomixis has occurred, the plants of the F_2 will show traits in a 3:1 ratio, and various combinations of parental characteristics, due to segregation of traits during meiosis. However, if apomixis has occurred, the resulting F_2 will substantially all be phenotypically identical to the F_1 generation. Additionally, there will be a number of male sterile plants, many of which will set seed. The existence of both these characteristics indicates that apomixis is occurring and that the seed being produced is identical to that produced by the original hybrid cross.

The AMS/vector can also be valuable as a plant vector system. The 40-110 nm (approx.) particles associated with extracts containing the AMS/vector have potential utility as intracellular plant delivery systems, e.g., for delivery of bioactive molecules such as nutrients, pesticides, etc. The 1×10^6 (approx.) dalton nucleic acid associated with extracts containing the AMS/vector, or a derivative, mutant, or fragment thereof, also has potential value as a transmissible expression vector. The nucleic acid, when comprised of a heterologous gene sequence, can be used as a vehicle for the expression of the heterologous gen sequence. Such a

nucleic acid can be used either in conjunction with, or without, the 40-110 nm particle.

6. EXAMPLES

6.1. SCREENING FOR AMS/VECTOR DONORS

Sterile alfalfa lines (obtained from the Seed Increase Collection, U.S.D.A., Reno, Nevada) were screened for the presence of the AMS/vector by a grafting experiment. Seventeen sterile alfalfa lines were first identified by visual ratings and acetocarmine staining for pollen, and then confirmed as steriles by crossing with alfalfa plants that later proved to be maintainers. Fifteen grafts for each of the sterile lines were performed, using different maintainer plants as scions (the upper part of the graft). The 255 grafts were placed in a mist chamber, were allowed to flower, and were selfed (by tripping). The seeds were harvested. In no cases was sterility observed in the graft generation. Plants of the next generation were germinated, and rated at flowering for the presence or absence of sterility. The flower was tripped, and rated as 1, 3, 5, or 7, according to the following:

1 = no anthers, no dehisced pollen
3 = anthers present, no dehisced pollen
5 = anthers present, dehisced pollen present
7 = anthers present, abundance of dehisced pollen present

That is, ratings of 1 or 3 meant the plant was sterile; ratings of 5 or 7 meant the plant was fertile.

In order to confirm plant sterility, attempts were made to self the sterile plants. Sterile plants were also crossed to a different maintainer plant in order to

prevent confounding sterility with inbreeding depression. The sterile plants were also crossed to restorer line Indiana Synthetic (C). Two criteria had to be satisfied in order to consider the plant an AMS/vector donor: (i) the maintenance of sterility after the cross with an unrelated maintainer plant; and (ii) the production of fertile progeny after the cross to the restorer line. Out of the 17 sterile lines screened, 5 lines were identified as AMS/vector donors.

The five identified AMS/vector sources were used again in a grafting experiment. Medicago scutellata, an annual cleistogomous (i.e., a plant that self-pollinates before flowering) that is very similar to soybean, was grafted on as scion for each of the five AMS/vector lines. The grafts did not alter fertility in the graft generation, which was all fertile; the next generation, however, contained male sterile plants at a frequency of approximately 10%.

6.2. CHARACTERIZATION OF NUCLEIC ACIDS ASSOCIATED WITH AMS/VECTOR DONORS

Alfalfa plants which screened positive for the AMS/vector, as discussed in Section 6.1., possess a unique nucleic acid. When extracts of such plants are applied to recipients (maintainers), the same nucleic acid is subsequently extractable from the recipient (now asexually induced to male sterility). The nucleic acid is not extractable from untreated isogenic maintainers.

This nucleic acid has a molecular weight of approximately 1.1×10^6 daltons (about 3.3 to 3.5 kilobases) and is postulated to be DNA. By the following procedure for DNA and RNA extraction from whole plants, the unique nucleic acid has been isolated from leaves, stems and/or primary callus tissue derived from ovules of the alfalfa plants described in Section 6.1. The same

procedure has been performed on alfalfa, soybean, and corn plants induced to mal sterility by tr atment with AMS/vector extracts and the unique nucleic acid was isolated from these plants as well.

To 5 g of plant tissue in a 50 ml centrifuge tube, 10 ml of 1x STE extraction buffer, 0.1 M sodium chloride (NaCl), 0.05 M Tris, 0.001 M ethylenediamine trichloroacetic acid (EDTA), pH 7.0, containing 1% mercaptoethanol is added. The tissue is ground in a Tekmar blender for one minute at 4°C. An additional 10 ml of boiling 1x STE extraction buffer containing 1% mercaptoethanol is then added, whereupon the tube is transferred to a 55°C water bath and stirred manually until the temperature reaches 50°C.

At this point, an equal volume (20 ml) of a 24:1 chloroform:isoamyl alcohol mixture is added and the tube is centrifuged for 10 minutes at 13,000 rpm with a Beckman J21C rotor, in a Sorvall centrifuge at 10°C. The resultant aqueous phase is removed to a new tube to which a volume, equal to one-tenth that of the aqueous phase, of 10% cetyltrimethylammonium bromide (CTAB) solution is added, followed by 20 ml of the 24:1 chloroform:isoamyl alcohol mixture. Again, the tube is centrifuged for 10 minutes at 13,000 rpm.

After centrifugation, the resultant aqueous phase is transferred to a new tube to which an equal volume of STE buffer is added. The tube is allowed to stand at room temperature (about 25°C) for 30 minutes and is then centrifuged for 5 minutes at 4,000 rpm in a Beckman J21C rotor. The supernatant fraction is removed and the remaining pellet is dried under a stream of nitrogen. The pellet can be stored frozen at -20°C until needed.

Next, the pellet is resuspended in 5 ml of a solution of 50 mM Tris, pH 8.0; 5 mM EDTA; 50 mM NaCl and

200 micrograms per ml (hereinafter "ug/ml") ethidium bromide. To this is added 4.4 g of cesium chloride (CsCl). The resulting mixture is centrifuged for 10 minutes at 28,000 rpm in a Beckman J21C rotor, and the clear supernatant fraction is retained.

Three ml of this supernatant are transferred to centrifuge tubes for a Beckman ultracentrifuge with an SW50.1 rotor and adjusted to 1.390 refractive index with CsCl. Two and a half ml of mineral oil are added to balance the tube to 6.1 g per tube. The tube is spun to equilibrium in a SW50.1 rotor for 60 hours at 23`C at 33,000 rpm. From this, the DNA fraction is removed.

The ethidium bromide is removed from the DNA fraction with three extractions of equal volumes of isopropanol equilibrated with 20x SSC, 0.15 M NaCl, 0.015 M sodium citrate, pH 6.8. The DNA fraction is diluted two-fold with a solution of 10 mM Tris, pH 7.6 and 1 mM EDTA, adjusted to 0.3 M sodium acetate. The DNA is precipitated with two volumes of ethanol. The precipitate is frozen at -20`C until further use.

The RNA pellet which results after the above-described 60 hour spin is resuspended in 0.5 ml of the 10 mM Tris, pH 7.6, 1 mM EDTA buffer and the ethidium bromide is extracted with two extractions of equal volumes of isopropanol equilibrated with 20x SSC. The RNA is diluted two-fold with 10 mM Tris, pH 7.6, 1 mM EDTA, adjusted to 0.3 M sodium acetate. Two volumes of ethanol are added and the mixture is frozen at -20`C until further use.

The DNA and RNA samples are thawed. Tubes with RNA are centrifuged for 10 minutes at 4`C at 10,000 rpm in a Beckman J21C rotor. The supernatant fractions are poured off. The RNA pellets remaining in the tubes are allowed to dry under a stream of nitrogen. Each RNA pellet is resuspended in 250 microliters (hereinafter "ul") of Tris borate buffer, 0.089 M Tris, 0.089 M boric

acid, 2.5 mM EDTA, pH 8.3, and a few drops of 0.1 M sodium acetate and then one volum of ethanol is added. These mixtures are frozen at -20°C until n eded.

The thawed DNA sampl s are transferred to 45 ul centrifuge tubes. Five ul of the Tris 10 mM, pH. 7.6 1 mM EDTA buffer are added and mixed until the precipitate dissolves. Ten ul of ethanol and a few drops of 0.1 M sodium acetate are added. No CsCl precipitate is observed. The DNA solutions are frozen at -20°C until needed.

The RNA samples are again thawed and centrifuged in a desk-top Eppendorf centrifuge for 3 minutes. The supernatant fractions are poured off and the pellets in the tubes are allowed to dry under a stream of nitrogen. The pellets are resuspended in 8 ul Tris borate buffer and 20 ul glycerol/dye (bromophenol blue) mixture. The samples are mixed well and stored at -20°C until needed.

The DNA samples are again thawed. They are centrifuged for 10 minutes at 4°C at 10,000 rpm in a Beckman J21C rotor, and resulting supernatant fractions are poured off. The pellets are dried under a stream of nitrogen and then resuspended in 5 ml of 10 mM Tris, pH 7.6, 1 mM EDTA buffer to which sodium acetate is added to a concentration of 0.3 M. Then 10 ml of ethanol are added. The DNA is allowed to precipitate at -70°C for one hour.

These DNA samples are thawed once more and centrifuged for 10 minutes at 4°C at 10,000 rpm in a Beckman J21C rotor. Supernatant fractions are poured off and the remaining pellets are dried under a stream of nitrogen. The pellets are resuspended in 500 ul Tris borate buffer and these mixtures are transferred to 1.5 ul microfuge tubes. To each tube are added a few drops of 0.3M sodium acetate and 1 ml of ethanol. The samples are frozen overnight at -20°C.

These DNA samples are again thawed and centrifuged in the desk-top Eppendorf c ntrifuge for 3 minutes. Supernatant fractions are poured off and pellets are dried under a stream of nitrogen. The pellets are resuspended in 80 ul Tris borate buffer. Twenty ul of glycerol/dye mixture are added and mixed well.

DNA and RNA samples so prepared are dialyzed overnight and run on a 1% agarose, 1x Tris-Borate-EDTA (TBE) gel. The DNA and RNA are pooled before running the gel. After ethidium bromide staining, the band characteristic of plants carrying the AMS/vector is seen at approximately 3.5 kb.

Photographs of such a gel from the experiment described supra is presented in Figs. 1A, 1B, 1C, and 1D. Fig. 1A depicts the 3.5 kb band present in alfalfa AMS/vector source 1.29 (U.S.D.A. PI No. 223386), and the absence of the 3.5 kb band in fertile untreated alfalfa maintainer (variety Arc) and fertile untreated non-maintainer (variety Arc). Fig. 1B depicts the 3.5 kb band present in alfalfa (variety Arc) converted to male sterility by treatment with AMS/vector source 1.29 (U.S.D.A. PI No. 223386). Fig. 1C depicts the 3.5 kb band present in corn (variety B73) converted to male sterility by treatment with AMS/vector source 1.26 (U.S.D.A. PI No. 221469). Fig. 1D depicts the 3.5 kb band present in soy (variety Williams 82) converted to male sterility by treatment with AMS/vector source 1.36 (U.S.D.A. PI No. 243223).

The approximately 3.5 kb nucleic acid associated with extracts containing the AMS/vector appears to be comprised of DNA (Fig. 1E). This was shown by digesting nucleic acid samples from alfalfa, prepared as described supra, with deoxyribonuclease (DNase, Boehringer Mannheim, 3000 U/mg) or ribonuclease (RNase, Boehringer Mannheim, 3000 U/mg). 20 ul of DNase or RNase (10 U/ul in 50 mM

NaCl, 50% glycerol) was added to 60 ul nucl ic acid sample, and the mixture was placed at 37°C for 15 minutes. The reaction was stopped by adding 15 ul of 0.4 M EDTA befor subjecting samples to agarose gel electrophoresis. The resulting ethidium bromide-stained bands are shown in Fig. 1E. The approximately 3.5 kb band associated with AMS/vector extracts is discernible in the RNase-treated samples, but is absent from the DNase-treated samples. The 3.5 kb nucleic acid thus appears to be comprised of DNA, as evidenced by its susceptibility to DNase digestion.

6.3. ELECTRON MICROSCOPY OF AMS/VECTOR PARTICLES

The following procedure was used to obtain electron micrographs depicting the 40-110 nm particles associated with the AMS/vector 1.29 (PI No. 223386).

All steps were carried out at 4°C or on ice. Buffer I consisted of: 50 mM Tris-HCl (pH 7.5), 0.4 M sucrose, 10 mM KCl, 5 mM $MgCl_2$, 10% (v/v) glycerol, and 10 mM 2-mercaptoethanol. Buffer II consisted of 50 mM sodium phosphate buffer, pH 7.0.

A sample of plant tissue was homogenized in a Virtis homogenizer in 6 volumes (v/s) of Buffer I for 30 seconds on slow speed and 30 seconds on fast speed. The homogenates were filtered through 4 layers of Miracloth and centrifuged at 2,000 x g for 5 minutes. The supernatant was centrifuged at 20,000 x g for 20 minutes. The resulting supernatant was centrifuged at 180,000 x g for 60 minutes in two tubes.

For further purification, the small, dark-green pellet was resuspended in 1.5 ml of Buffer II and layered onto a 12-42% (w/w) gradient of sucrose in Buffer II. Gradients were centrifuged at 35,000 rpm in an SW41 rotor at 4°C for 75 minutes. There were no visible bands in the

gradient. Approximately 0.5-ml fractions wer removed from the tops of the tubes, and ach fraction was diluted to 0.8 ml for determination of OD_{254}. Fractions from the shoulder region at the leading edge of the peak (at about one-third the distance from the top of the tube) were pooled and stored at 4°C overnight. Samples were dialyzed against Buffer II to remove the sucrose, and were then centrifuged in an SW50.1 rotor at 40,000 rpm for 75 minutes.

For negative staining, the small white pellet was suspended in about 0.2 ml of Buffer II. Five microliters of the suspension was placed on a Formvar-coated grid and allowed to sit for about 2 minutes. Excess liquid was washed off and the grid (sample side down) was floated on a drop of 2% uranyl acetate for 2 minutes. Excess liquid was washed off.

The sample was examined by transmission electron microscopy. Some vesicle-like particles were seen, several with dense cores, but these did not have sharply defined structures. Some micrographs, taken of the AMS/vector source male-sterile line, appeared to depict 40-110 nm particles. Figure 2A shows the 40-110 nm particles present in a crude extract (prepared as herein described) of a male-sterile alfalfa plant, AMS 1.29 (PI No. 223386). Figure 2B depicts the 40-110 nm particles present in an ovule of a male-sterile alfalfa plant, AMS 1.26 (PI No. 221469). Figure 2C depicts a thin section of a seed from a cross between an alfalfa maintainer plant and a formerly fertile alfalfa plant that was converted to male sterility by treatment with extracts of AMS/vector source 1.29. The white inclusion bodies exhibiting dark spots may contain the approximately 3.5 kb nucleic acid associated with extracts of the AMS/vector.

6.4. INDUCTION OF MALE STERILITY IN ALFALFA

The experiment described herein demonstrates the asexual induction of male sterility in alfalfa, mediated by the AMS/vector.

The experimental design was a randomized complete block design, as a split-plot, with treatments as the main plot, and varieties as splits. There were four replications, with 16 treatment plots within each replicate, and 8 genotypes (varieties) randomly distributed as 1 of 8 rows in each plot. The 16 treatments and 8 genotypes tested are listed in Tables IA, IB.

TABLE IA.

EXPERIMENTAL TREATMENTS OF ALFALFA

Code	Treatment
T1	Injection, source 1.7 AMS/vector
T2	Injection, Source 1.4 AMS/vector
T3	Injection, 1.26 AMS/vector
T4	Injection, source 1.36 AMS/vector
T5	Injection, source 1.29 AMS/vector
T6	Injection, Indiana Synthetic (C) (alfalfa restorer)
T7	Injection, maintainers isolated from Arc variety of alfalfa
T8	Injection, buffer only (KH_2PO_4, pH 6.9)
T9	Celite application, source 1.7 AMS/vector
T10	Celite application, source 1.4 AMS/vector
T11	Celite application, source 1.26 AMS/vector
T12	Celite application, source 1.36 AMS/vector
T13	Celite application, source 1.29 AMS/vector
T14	Celite application, Indiana Synthetic (C) (alfalfa restorer)
T15	Celite application, maintainers isolated from Arc variety of alfalfa
T16	Celite application, buffer only (KH_2PO_4, pH 6.9)

TABLE IB.

ALFALFA GENOTYPES

Genotype Number	Genotype Descriptions
1	Source 1.26, AMS/vector
2	Source 1.36, AMS/vector
3	Source 1.29, AMS/vector
4	Fertile maintainer
5	Fertile maintainer
6	Fertile maintainer
7	Fertile maintainer
8	Indiana Synthetic (C), restorer

U.S.D.A. Plant Introduction (PI) Nos. for each AMS/vector source are listed in Table IC.

TABLE IC.

U.S.D.A. PLANT INTRODUCTION NUMBERS OF ALFALFA AMS/VECTOR SOURCES

AMS/Vector Source Designation	Plant Introduction Number
1.4	172429
1.7	173733
1.26	221469
1.36	243223
1.29	223386

Treatments consisted of injection of soluble extracts, or Celite (diatomaceous earth, grade III, Sigma Chemicals, Cat. No. D5384) application. Injections were done with a 28-gauge needle. The needle was passed through the stem of the plant, a drop of extract was exuded on the other side, and the needle was pulled back through the stem. Approximately 5 stems were injected per plant. Any stem that was not injected was trimmed back. Celite application was carried out basically as described in Section 6.9.1.6.3, infra.

Each genotype was rated for sterility three weeks after treatment. Flowers were tripped on emory cloth, and scored as follows:

1 = no anthers; no pollen
3 = anthers present; no pollen
5 = anthers present; small amounts of pollen present
7 = anthers present; sufficient pollen present

A rating of 1 or 3 was consid red a sterile; 5 or 7 was considered a fertil . Acetocarmin staining was used to confirm the visual ratings.

The r sults r v al d that treatments T1 through T5, and T9 through T13 (all AMS/vector sources) altered the fertility of genotypes 4 through 7 (maintainers) from a 7 down to a 3, i.e., converted the fertile maintainers to a sterile state. Analysis of variance indicated that the treatment effects were highly significant at P = 0.01, and that the fertility within the treated generation of the maintainers had been affected and was not significantly different from the AMS/vector sources (genotypes 1-3). As expected, genotype 8 (restorer) remained fertile with all treatments, and genotypes 1-3 (AMS/vector sources) retained sterility with all treatments. Any variation in replication results was not significant.

6.5. INDUCTION OF MALE STERILITY IN SOYBEAN

The experiment described herein demonstrates the asexual induction of male sterility in soybeans, mediated by the AMS/vector.

The experimental design was a randomized complete block design, a split-plot, as described in Section 6.4, supra. Treatments T1 through T5, and T9 through T13, were as described in Section 6.4. Treatments T6 and T14 were injection and Celite application, respectively, of extracts of the particular genotype being treated. Treatments T7 and 15 were untreated plants. Treatments T8 and T16 were injection and Celite application, respectively, with buffer (KH_2PO_4, pH 6.9) only. Injections were done at the nodes and into the petioles (stem tissue between the stem and leaf) of each

plant. The eight genotypes treated are listed in Table ID.

TABLE ID.

SOYA GENOTYPES

Genotype Number	Variety Name
1	Williams 82
2	Wells II
3	Century
4	Hobbit
5	Cumberland
6	McCall
7	Traff
8	Maple Presto

Fertility was rated as described in Section 6.4, supra.

The results showed that treatment with all AMS/vector sources (T1 through T5; T9 through T13) affected the fertility of Genotypes 1, 2, and 3, with 20% sterility observed in Williams 82, and 17-30% sterility observed in both Wells II and Century.

Analysis of variance indicated a significant treatment effect (P at 0.01). Any variation in replication results was not significant. Genotypes 4-8 were not altered in their fertility. There were no differences observed between treatment by injection or Celite application.

6.6. ADDITIONAL DATA ON INDUCTION OF MALE STERILITY IN SOYBEAN

Seeds of male (purple flowers; Wells II) and female (white flowers; Williams 82) soybeans were planted. 27 and 35 days later, a total of 186 female and 24 male plants, at approximately the three-internode stage, were sprayed with AMS/vector extract from alfalfa AMS1.4 or AMS1.36 lines. Spray applications were carried out with Celite, in KH_2PO_4, pH 6.9 (see Section 6.10.1.6.3, infra).

9-10 days and 19 days after the last spraying, treated soybean plants producing flowers were rated for fertility based on the presence or absence of pollen in anthers excised from flowers. At least three flowers per plant were rated.

In all cases, all flowers examined produced large amounts of pollen (fertile) or no pollen of any kind (sterile). This "black and white" rating was in contrast to the situation in alfalfa lines in which sterile plants exhibit a range of phenotypes (e.g., no pollen, reduced amount of pollen, aborted pollen, or pollen not released from anthers).

The results of the induction of sterility in soybean are presented in Table IE.

TABLE IE.

AMS/VECTOR-INDUCED STERILITY IN SOYBEANS

	Percent Sterility Observed in Treated Soybean Plant	
Soybean Recipient	AMS/Vector Source AMS1.36	AMS/Vector Source AMS1.4
White female	19/49 (39%)	35/126 (25%)
Purple male	2/10 (20%)	6/14 (43%)

The results shown in Table IE indicate that both AMS/vector source extracts were capable of inducing male sterility, with the purple-flowered soybean line responding more strongly to the AMS1.4 extract, while the white-flowered line responded more strongly to the AMS1.36 extract. 50 of the female (white-flowered) plants out of a total of 175 rated were sterile, and 8 of the male (purple-flowered) plants out of a total of 24 rated were sterile. Therefore, sterility was induced in 58 out of 199 or 29% of the plants rated.

6.7. INDUCTION OF MALE STERILITY IN CORN

The experiment described herein demonstrates the asexual induction of male sterility in corn. The experimental design and treatments 1-5 and 9-13 were as described in Section 6.4, supra. Treatments 6 and 14 were injection and Celite application, respectively, of extracts of the particular genotype being treated. Treatments 7 and 15 were untreated plants. Treatments 8 and 16 were injection and Celite application,

respectively, with buffer alone. Celite applications were carried out as described in Section 6.9.1.6.3, infra. Injection was by us of a 28 gauge needle inserted into the pith of the plant. Genotypes which were subjected to experimental treatments are listed in Table IF.

TABLE IF.

CORN GENOTYPES

Genotype Number	Genotype Name
1	B73*
2	A632*
3	Mo17*
4	Mo17 (Indiana Crop Improvement Association)
5	B73 (Indiana Crop Improvement Association)
6	VA26*
7	H84*
8	H95*

*, Obtained from the Purdue Agricultural Experiment Station.

Fertility was rated according to the following:

1 = deformed anthers present; no pollen
3 = normal anthers present; no pollen; no stainability with acetocarmine

5 = normal anthers present; stainable pollen present

7 = normal anthers present; abundant pollen present

The results showed that the fertility of genotypes 1 through 7 was altered with all treatments with AMS/vector sources. The range of sterility conversion was 15-26% for genotypes 1-7, with treatment effects being highly significant (P less than 0.01). There was no observed effect of treatments on genotype 8. Any variation in replication results was not significant.

6.8. INDUCTION OF MALE STERILITY IN OTHER PLANTS

Observational tests were conducted to evaluate the inducibility of male sterility mediated by the AMS/vector in sorghum, sunflower, pearl millet, and tomato. Treatment with AMS/vector sources appeared to result in reduced seed set in sorghum, sunflower, and millet, and reduced fruit set in tomatoes.

6.9. INDUCTION OF APOMIXIS IN SOYBEAN

Hybrid crosses were initiated using an AMS/vector-treated, male sterile, white-flowered "Williams-82" soybean line as female parent, and normal pollen producing fertile, purple-flowered "Wells-II" as a male parent. The F_1 generation produced, as expected, consisted entirely of purple flowered, purple hypocotyl plants. This F_1 generation was selfed to produce plants of the F_2 generation, which were in turn used as the basis of an inheritance study through the F_5 generation. These plants were examined for flower and hypocotyl color, anther and pollen characteristics (male sterility) and podding status.

6.9.1. HYPOCOTYL AND FLOWER COLOR

Plants having a purple hypocotyl had purple flowers and those having a green hypocotyl had white flowers. There was a predominance of purple hypocotyls and purple flowers in all four generations (F_2-F_5). Thirty-seven of the 38 plants rated in F_2 (97.4%), 38 of the 39 plants in F_3 (97.4%), 39 of the 40 plants in F_4 (95%), and 22 of the 32 plants in F_5 (68.8%) had purple hypocotyls and purple flowers. The percentages of plants that bore green hypocotyls and white flowers in the F_2, F_3, F_4 and F_5 generations were 2.6%, 2.5%, 5%, and 31.2%, respectively.

6.9.2. CHARACTERISTICS OF MALE STERILITY

Two flowers from each plant of F_2-F_5 generations were examined. Anther, pollen and stigma were stained with acetocarmine and examined under a microscope. Two categories of flowers were observed. In one category, characteristics of anther, pollen, and stigma were very typical of descriptions in the literature for fertile soybean flowers (Albertsen and Palmer, Am. J. Bot. 66:253-265, 1979). Dehiscence of anthers was complete, anthers encircled the stigma, and the dehisced pollen from the anthers was deposited on the stigmatic surface. Occasionally, pollen tubes were seen on the stigmatic surface Pollen was uniform in size and shape, and stained deep red with acetocarmine. Flowers or plants bearing such flowers belonging to this category were designated as bearing normal pollen.

With a few exceptions of partial dehiscence, the anthers did not dehisce, in the second category of flowers. Pollen grains remained in the anther, and could be liberated only when the anther was crushed. Pollen grains were not uniform in size and shape, and looked abnormal. A mixture of stainable and non-stainable pollen

grains was apparent. The non-stainable pollen varied in size and shape, was highly vacuolated, and did not have a well defined pollen wall. The stainable pollen was round in shape and looked abnormally large (compared to normal dehisced pollen grains). In a number of preparations, pollen wall development was found to be irregular and incomplete. Occasionally cytoplasm was seen oozing out of such pollen grains. No pollen grains were detected in anthers of two plants belonging to F_5 generation. Pollen grains were not seen on the stigmatic surface. Flowers or plants bearing such flowers were designated "male-sterile".

Typically, both flowers from each plant fell into either one or the other of these categories. In subsequent descriptions plants were either designated "normal-pollen-bearing" or "male-sterile".

Remarkable consistency was observed in the morphological features of anthers, pollen and stigma in each of these two categories, across all the four generations.

6.9.3. FREQUENCY OF MALE STERILITY IN F_2-F_5 GENERATIONS

Most of the plants in each generation bore "sterile pollen" and were designated "male-sterile". Twenty-nine of the 38 plants rated in F_2 (76.3%), 29 of the 39 plants in F_3 (74.4%) and 31 of the 40 plants in F_4 (77.5%)pand 18 of the 32 plants in F_5 (56.2%) were "male-sterile". As evident from this data male sterility in the F_2, F_3, and F_4 generations was higher than in the F_5 generation. These results are summarized in Table IG.

6.9.4. PODDING STATUS

All plants rated as "normal-pollen-bearing" formed a number of seed-bearing pods across all the four

generations (Table IH). A majority of the pods in this category had 3 seeds per pod, with 2 seeds and 1 seed per pod being of rare occurr nce.

A number of plants rat d as "male-st rile" also formed seed-bearing pods. Ten out of 29 plants in F_2 (34%), 18 out of 29 plants in F_3 (62%), 18 out of 31 plants in F_4 (58%) and 9 out of 18 plants in F_5 (50%) had seed-bearing pods. A majority of the plants in this category had three seeds per pod. In some plants one seed per pod and two seeds per pod were also common.

The rest of the "male-sterile" plants produced a large number of flowers but had no pods. Percentages of "male-sterile" plants that had no pods in F_2, F_3, F_4 and F_5 generations were 14, 3, 3 and 6, respectively.

6.9.5. SEED VIABILITY

A sample of seed from each of the four generations was collected from the mature pods, and germinated on moist filter paper. Germination was more than 99% overall. Seed produced from both normal pollen bearing plants in F_2-F_5 generations and "male-sterile" plants in the F_2-F_4 generations, had excellent seed viability. Seeds from male-sterile plants of the F_5 generation were not mature when the germination test was conducted.

TABLE IG.

SUMMARY OF DATA FOR HYPOCOTYL COLOR, FLOWER COLOR AND MALE STERILITY IN PLANTS BELONGING TO F_2, F_3, F_4 AND F_5 GENERATIONS.

	F_2	F_3	F_4	F_5
Number of Plants Rated	38	39	40	32
Plants with Purple Hypocotyl and Purple Flowers	37 (97.4%)*	38 (97.4%)	38 (95%)	22 (68.8%)
Plant with Green Hypocotyl and White Flowers	1 (2.6%)	1 (2.6%)	2 (5%)	10 (31.2%)
Plants Showing Normal Pollen	9 (23.7%)	10 (25.6%)	9 (22.5%)	14 (43.8%)
Plants Showing Sterile Pollen ("Male-Sterile")	29 (76.3%)	29 (74.4%)	31 (77.5%)	18 (56.2%)

*Percentage of total plants rated in parentheses.

TABLE IH

RELATIONSHIP[+] BETWEEN FLOWER RATING AND PODDING STATUS IN F_2 - F_5 PLANTS

	Generation			
	F_2	F_3	F_4	F_5
Number of Plants Rated	38	39	40	32
Plants with normal pollen	9	10	9	14
Plants containing pods with seed	9 (100%)*	10 (100%)	9 (100%)	14 (100%)
Plants with sterile pollen	29	29	31	18
Plants containing pods with seed	10 (34%)**	18 (62%)	18 (58%)	9 (50%)
Plants with no pods	4 (14%)**	1 (3%)	1 (3%)	1 (6%)

+ Relationship between flower rating and podding status expressed as percentages, shown in parentheses for each of the two categories of normal pollen and sterile pollen rated plants, itemized separately in the two horizontal columns in the table.

* Percentage of normal pollen rated plants.

** Percentage of sterile pollen rated plants.

6.9.6. RESULTS AND DISCUSSION

The foregoing results were consistent with a pattern of apomictic reproduction of hybrid seed, in that there has been demonstrated a failure of segregation in the F_2 and subsequent hybrid generations, and setting of seed in male sterile plants. This pattern has been shown to be maintained over several generations. Although not wishing to be bound by any theory, it may be that the breakdown in apomixis which has been observed reflects an original induction of a facultative type of apomixis, such as occurs routinely in nature among certain types of apomictic plants. However, any reversion back to normal reproduction can be cured by a reapplication of the AMS/vector to the original hybrids.

6.10. FIELD TEST OF AMS/VECTOR TREATMENTS ON CORN PLANTS

The examples described herein demonstrate the induction of male sterility, mediated by the AMS/vector, in corn grown under field conditions.

Four treatments involving AMS/vector sterility sources and four control treatments were applied to corn, grown under field conditions, to evaluate the effect of the AMS/vector (extracted from alfalfa) on induction of male sterility in corn. Four varieties (inbreds) of corn were used in this study to examine variety-AMS/vector interactions. Pollen presence or absence was noted. Pollen stainability, plant height, ear height, and time to 75% silking were also observed to determine if there might be any other treatment effects such as plant growth stimulation. The statistical significance of treatment differences was assessed using analysis of variance, and Duncan's multiple range tests.

Th re was a strong treatment effect for the pollen sterility (pollen absenc or presence) variable.

Analysis of variance showed significant treatment differences at P less than 0.0001 for this character. The analysis of variance also showed a strong variety effect. Comparison among treatment m ans was conducted for three varieties of corn (varieties 1, 2, and 4 of Table II, infra), in which there was strong evidence (P less than 0.0001) that corn plant sterility was effected by the treatments used in the experiment. There was no statistically significant evidence of a treatment effect in variety 3. Duncan's multiple range test revealed that the means of treatments with corn extract (no AMS/vector), buffer only, alfalfa extract (no AMS/vector), and untreated plants were significantly different from that of sources 1, 2, 3 and 4 AMS/vector treatments. In variety 1, means for source 4 AMS/vector treatment were different from the rest, while in variety 4, means for source 2, AMS/vector treatment were different from the rest.

The analysis of variance for plant height indicated significant (P less than 0.0106) treatment differences and also differences among the four varieties of corn. Duncan's multiple range test indicated that all comparisons of plant height between any two varieties were significantly different at P less than 0.05.

The analysis of variance results for ear height did not reveal any treatment differences for this character. However Duncan's multiple range test revealed that ear height of corn varieties were significantly different from each other.

No treatment effect was evident in days to 75% silking in any of the corn varieties. However, both analysis of variance and Duncan's multiple range test revealed a significant difference among the four corn varieties for this character.

Treatments with sources 1, 2, 3, and 4 AMS/vector showed pollen sterility in corn varieties 1, 2,

and 4. Treatments with corn xtract (no AMS/vector), buffer alone, alfalfa extract (no AMS/vector), and untreated plants showed no pollen sterility in any corn variety. Variety 3 showed no pollen sterility under any treatment. There was a position effect in the expression of sterility, with plants showing sterility occurring in clusters among the fertile plants.

Microscopic examination of one flower from each of the plants with no dehisced pollen (pollen sterility), revealed that varieties 1 and 2 had non-stainable, abnormal, and irregular pollen inside their anthers, which never dehisced. Variety 4 did not produce any pollen, nor did the anthers dehisce. Pollen from all plants in variety 3, and from fertile plants of varieties 1, 2, and 4 showed normal, round, stainable pollen typical of untreated corn plants.

6.10.1. MATERIALS AND METHODS

6.10.1.1. CORN SEED SOURCE

Four corn varieties (described in Table II) were selected for this study. The varieties were obtained from the Ohio Foundation Seed Company, Croton, Ohio. The four varieties were received and the identities recorded, by field site personnel. The varieties were coded and provided as knowns to the investigating team. The variety codes are listed in Table II.

TABLE II.

CORN PROJECT VARIETY CODES

Corn Seed Source	Variety
A632Ht, Lot 950, Grade F	1
B73Ht, Lot 4551 ST, Grade 23-21F	2
H95Ht, Lot 150, Grade MF	3
M017Ht, Lot 055, Grade MF	4

The seed was stored in the cold room at 38°F until the time of planting.

6.10.1.2. FIELD PREPARATION

The experiment was conducted in a field site in West Jefferson, Ohio. The field site (150 feet x 120 feet) was plowed, disked and rototilled. A basal fertilizer application was made using a fertilizer applicator and consisted of P_2O_5 (28 lbs.), K_2O (39 lbs.), and urea (140 lbs.), as phosphorus, potassium, and nitrogen sources, respectively. Another dose of 140 lbs. of urea was applied between rows, by hand, four weeks after emergence. A preemergence herbicide, Lasso (Monsanto, St. Louis), was applied to control the weeds. The field was rototilled at a shallow depth of four inches again after the fertilizer and herbicide application. The experimental plot was surrounded on two sides by ecology experiments and on two sides by fields leased to farmers. Those fields were in oats for the period of the experiment.

6.10.1.3. EXPERIMENTAL DESIGN

The design used was a split-plot with tr atments as the main plot and varieties as splits. There were four replications, with eight treatment plots within each replicate and four varieties randomly distributed as one of four rows in each plot. Each variety was planted as a 20 foot row, with 6 inches in-row and 3 feet between-row spacing. All plots were separated by a matrix of 6 foot wide alleys. The treatment and variety randomizations were as outlined in Table III.

TABLE III.

CORN TREATMENT AND VARIETY RANDOMIZATIONS

	T7	T6	T3	T8	T4	T5	T2	T1
R1	4231	1243	1234	4213	4132	1324	3142	2341
	T8	T4	T7	T2	T1	T6	T5	T3
R2	1432	4213	1243	2143	3214	4312	4213	2413
	T2	T6	T1	T5	T3	T8	T4	T7
R3	1342	3412	4132	1423	4132	2134	2431	3412
	T5	T2	T8	T1	T3	T4	T7	T6
R4	4321	1243	4312	4123	3124	1243	2341	3241

The eight treatments used (T1 through T8) in each replication are as listed in Table IV.

TABLE IV.

CORN PROJECT TREATMENT CODES

Treatment	Code 1	Code 2 (Field Site Code)
Corn extract (no AMS/Vector)	T1	B2
Source 2, AMS/Vector (U.S.D.A. PI No. 172429)	T2	B6
Source 1, AMS/Vector (U.S.D.A. PI No. 221469)	T3	B1
Alfalfa extract (no AMS/vector)	T4	B8
Untreated	T5	B7
Source 3, AMS/Vector (U.S.D.A. PI No. 223386)	T6	B4
Source 4, AMS/Vector (U.S.D.A. PI No. 243223)	T7	B5
Buffer only	T8	B3

6.10.1.4. CORN PLANTING

All four corn varieties were hand planted. Each variety was planted in a 20 foot row, with 6 inch spacing between each plant (i.e., forty plants in each row). The varieties were planted in conformance with the randomization chart (Table III).

6.10.1.5. COLLECTION AND SHIPMENT OF TREATMENT AND CONTROL MATERIALS

Alfalfa material was cut fresh from the field, immersed in liquid nitrogen, and the liquid nitrogen frozen material shipped overnight to the field site. The alfalfa material was used as a basis for five of the eight treatments, one of which was the control without AMS/vector

and four of which contained AMS/vector. The AMS/vector sources were four different mal sterile genotypes developed from four alfalfa lines, namely PI Nos. 221469, 172429, 223386, and 243223. The alfalfa control extract (without AMS/vector) was a maintainer isogenic non-sterile line.

The frozen alfalfa material arrived in sealed plastic bags, precoded as T1, T2, T3, T4, and T5 (see Table IV, supra). A record was retained elsewhere of the control and AMS/vector treatment materials and the corresponding codes on the plastic bags. The treatment codes were therefore "blind" for those who performed the field test.

6.10.1.6. PREPARATION AND APPLICATION OF AMS/VECTOR TREATMENTS AND CONTROL TREATMENTS

6.10.1.6.1. TREATMENT MATERIALS AND THEIR SOURCES

The alfalfa material received and stored frozen, was used to prepare four extracts containing AMS/vector and one alfalfa extract free of AMS/vector as a control. Material from four corn varieties were used to prepare a corn extract control. These varieties were untreated plants, planted in the same field as the full experiment, in eight rows, two rows per variety, each 90 feet long. Freshly collected plant tissue from each of these varieties was used to prepare the corn extract control treatment. The other two control treatments were one in which only buffer (0.067 M KH_2PO_4, pH 6.9) was applied, and another in which neither the buffer nor plant extract was applied (see Table IV, supra).

6.10.1.6.2. EXTRACTION PROCEDURE

Phosphate buffer (KH_2PO_4, 0.067 M, pH 6.9) was prepared three days before the extraction of the plant material and was stored at 11°C. All the extraction procedures were done wearing disposable surgical gloves. The

gloves were disposed of after the extraction of each treatment mat rial was completed. A new pair of gloves was used for each treatm nt.

The frozen alfalfa plant material was transferr d to the West Jefferson field facility in an ice box under dry ice. The ice box was kept in a cold room at 38°F until the extractions began. For each treatment extract, a total of 310 g of plant material and 2200 ml of buffer was used. Because of the availability of only one centrifuge, the extraction was done in two batches for each treatment, each using 155 g of plant material and 1100 ml buffer. The buffer and the plant material were macerated for 2-3 minutes in a Waring heavy duty blender. The homogenate was filtered through four layers of sterile cheesecloth to remove the plant debris. The filtrate was collected in sterile 250 ml centrifuge bottles and centrifuged at 2,000 rpm for five minutes at 11°C in a GSA rotor. The supernatant was decanted into sterile flasks, labeled, and put in the cold room at 38°F until used for spraying. Freshly collected leaf material from the four corn varieties was similarly extracted. The supernatants derived from the above procedure were used as the treatment materials for spraying corn plants.

6.10.1.6.3. APPLICATION OF EXTRACTS

Personnel conducting the field test were unaware of the identity of the treatments, and the treatment plots were coded by them (see Table IV, supra). Thus, the study was "double-blind."

Celite (diatomaceous earth, grade III, Sigma Chemicals Cat. No. D5384) was used as an abrador. One hundred grams of Celite was added to 1000 ml of KH_2PO_4 buffer (0.067 M, pH 6.9, 11°C), in a one gallon garden tank sprayer. The Celite-buffer mix was vigorously shaken, to ensure a uniform dispersion of Celite in the buffer for spraying.

Corn plants were sprayed when the fifth leaf was fully expanded, four weeks after planting. All plants were sprayed around the whorl (tip of corn plant) with Celite, using the one gallon tank sprayer. The six plant extracts and the buffer-only control were sprayed around the whorl using the one gallon tank sprayers. Treatment T5 plants received no buffer and no plant extracts.

6.10.1.7. COLLECTION OF DATA

Data collection included five parameters: pollen presence or absence, pollen stainability, plant height (inches), time (days) to 75% silking, and ear height (inches). The parameters plant height, time to 75% silking, and ear height were observed to determine if there might be any other effects of the treatment applications, such as plant growth stimulation. An average for plant height, days to 75% silking, and ear height was calculated for each row within each treatment plot. Photographs were taken depicting representative appearance of the pollen rating. Data collection for pollen absence or presence began when anthers first appeared on the tassel. An 8-1/2 inch x 11 inch black paper was placed below the tassel. At the time of pollen shed (7:30 AM - 11:30 AM), the plant was shaken twice. Plants were tagged (with colored twine) according to their pollen rating as follows:

1 = No dehisced pollen = orange tag
2 = Presence of dehisced pollen = yellow tag

Pollen stainability was rated in one of each of the two types of results, one each for each variety in each treatment. One fertile flower for each variety treatment was stained. A portion of the tassel with no dehisced pollen from the field was stain d with acetocarmine and observed for normal or abnormal appearance. Pollen staining was done by

transferring the anther to a glass slide, applying a drop of acetocarmine and covering th stam n with a coverslip. Pollen stainability was observ d immediately. Photographs of representative normal and abnormal microscopic fields were also taken.

6.10.1.8. STATISTICAL ANALYSIS OF THE DATA

Four dependent variables were analyzed separately using the tests described below. The four data variables were pollen amount, ear height, plant height, and days to 75% silking. The pollen stainability variable was not statistically analyzed because only one flower was stained in each category, namely flowers with dehisced pollen or with no dehisced pollen. The data and trends were tabulated for pollen stainability.

Data was analyzed for this split-plot design, with appropriate error terms using the Statistical Analysis System (SAS) program. Analysis of variance (F statistic) was used to test for statistically significant differences among the eight treatments. Analysis of variance determines if there is a significant difference between what is observed (treatments) and the expected random values (untreated). To draw inferences from any such differences requires that we have replications to enable us to calculate experimental error, and randomization to ensure a valid measure of experimental error. We satisfied both of these requirements by having four replications. The plants were randomized within the treatments and the treatments were randomized within each of the replications. Significance probabilities less than or equivalent to $P = 0.01$ are considered strong evidences in favor of a treatment effect.

Mean separations were done using Duncan's new multiple range test. Duncan's multiple range test does not in itself determine if there is a "significant difference" from the null hypothesis, but allows us to break our

treatments into groups that are significantly different from each other. For example, assume that tr atments T1, T2, T3 and T4 are all significant at P less than 0.05. Duncan's test allows us to determine if they are equally different (one class) or unequally different, e.g. T1 and T2 in class A, and T3 and T4 in class B. Treatment means were compared using critical range values.

6.10.2. RESULTS AND DISCUSSION

6.10.2.1. STATISTICAL ANALYSIS

The experimental design established for this study is called a split-plot design. The split-plot helps reduce error by keeping treatment blocks together. There is still randomization of plants within each treatment and of treatments within each replication. (A completely randomized design would not separate treatments into blocks). In our split-plot design, eight "whole" plots were selected so as to be linearly contiguous in space. One of eight treatments (some of which were controls) were randomly assigned to these whole-plot units. Each whole-plot unit was then subdivided or "split" into split-plots. Each split-plot unit was randomly assigned one of four varieties of corn plants to be planted in that split-plot. Finally, this design was replicated four times.

Five responses to the treatments were measured: number of sterile plants, number of fertile plants, plant height, ear height, and days to silking. Another response variable was created by dividing the number of sterile plants by the total number of sterile and fertile plants. This response variable is the percent of plants that are sterile. Since data expressed as percentages do not adhere to the assumption of constant response variation among plants treated alike, a variance stabilizing transformation was applied which allows for a more appropriate statistical

analysis. If in this experiment, p represents the percent sterile, then the appropriate transformation is as follows:

$$z = \sin^{-1}(p^{-1/2}) \qquad (1)$$

where z represents the new response variable used for analysis.

The means of percent sterile, plant height, ear height, and days to silking for each combination of treatment and variety are given in Tables V, XV, IX, XXIII, respectively, infra. Each mean is an average of the four replications. The means of percent sterile and days to silking were really "back-transformed" means. That is, each of these two variables was transformed (eq. 1), the average of the transformed values was computed, and then these averages were "back-transformed" to their original scale.

6.10.2.1.1. ANALYSIS OF POLLEN RATING (TABLES V THROUGH XIV)

Male sterility was observed only in treatments B1 (source 1 AMS/vector), B4 (source 3 AMS/vector), B5 (source 4 AMS/vector) and B6 (source 2 AMS/vector), in varieties 1, 2 and 4. No male sterility was seen in variety 3 (Table V).

TABLE V.

AVERAGE "% STERILE" RESPONSES FOR EACH STERILITY TREATMENT (1 - 8) FOR CORN VARIETIES 1 - 4.

	TREATMENT							
	B1	B2	B3	B4	B5	B6	B7	B8
	Source 1 AMS/Vector	Corn Extract (No AMS/Vector)	Buffer	Source 3 AMS/Vector	Source 4 AMS/Vector	Source 2 AMS/Vector	Untreated	Alfalfa Extract, (No AMS/Vector)
VARIETY	PERCENT STERILE							
1	17.05	0.00	0.00	15.97	9.94	18.10	0.00	0.00
2	42.10	0.00	0.00	39.11	37.60	29.33	0.00	0.00
3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4	21.87	0.00	0.00	22.16	20.65	14.72	0.00	0.00

The analysis of variance results for the percentage of corn plants that were male sterile show that there was a strong (P less than 0.0001) treatment eff ct. That is, it is highly improbable, less than 1 in 10,000, that the percentage of corn plants that became sterile in each treatment group is the same for every treatment group (Table VI).

TABLE VI.

ANALYSIS OF VARIANCE: TRANSFORMED PERCENT STERILE (ALL FOUR CORN VARIETIES).

SPLIT PLOT ANALYSIS OF CORN DATA

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: ASINQ

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR F	R-SQUARE	C.V.
MODEL	55	8.41029291	0.15291442	31.28	0.0001	0.959835	36.9605
ERROR	72	0.35193684	0.00488801		ROOT MSE		ASINQ MEAN
CORRECTED TOTAL	127	8.76222976			0.06991432		0.18915975

SOURCE	DF	TYPE I SS	F VALUE	PR F	DF	TYPE III SS	F VALUE	PR F
REP	3	0.00753203	0.51	0.6742	3	0.00753203	0.51	0.6742
TRT	7	4.61038623	134.74	0.0001	7	4.61038623	134.74	0.0001
REP*TRT	21	0.10714971	1.04	0.4261	21	0.10714971	1.04	0.4261
VAR	3	1.80852032	123.33	0.0001	3	1.80852032	123.33	0.0001
TRT*VAR	21	1.87670462	18.28	0.0001	21	1.87670462	18.28	0.0001

TESTS OF HYPOTHESES USING THE TYPE III MS FOR REP*TRT AS AN ERROR TERM

SOURCE	DF	TYPE III SS	F VALUE	PR F
REP	3	0.00753203	0.49	0.6916
TRT	7	4.61038623	129.08	0.0001

The analysis of variance results also show a strong (P less than 0.0001) variety effect. However, the magnitude of the difference between any two treatment means is not constant from one variety of corn plant to another. This latter effect, known as an interaction effect, suggests that comparisons among treatment means should be conducted within a single variety.

For varieties one, two, and four there is strong evidence (P less than 0.0001) that corn plant sterility was affected by the treatments used in the experiment. However, there is no statistically significant evidence of a treatment effect in variety three (Table VII, VIII, IX, and X).

TABLE VII.

ANALYSIS OF VARIANCE: TRANSFORMED PERCENT STERILE FOR CORN VARIETY 1.

SPLIT PLOT ANALYSIS OF CORN DATA

VAR=1

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: ASINQ

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR F	R-SQUARE	C.V.
MODEL	10	1.32087578	0.13208758	42.53	0.0001	0.952947	27.9215
ERROR	21	0.06522039	0.00310573		ROOT MSE		ASINQ MEAN
CORRECTED TOTAL	31	1.38609617			0.05572910		0.19959223

SOURCE	DF	TYPE I SS	F VALUE	PR F	DF	TYPE III SS	F VALUE	PR F
REP	3	0.01163036	1.25	0.3175	3	0.01163036	1.25	0.3175
TRT	7	1.30924541	60.22	0.0001	7	1.30924541	60.22	0.0001

TABLE VIII.

ANALYSIS OF VARIANCE: TRANSFORMED PERCENT STERILE FOR CORN VARIETY 2.

SPLIT PLOT ANALYSIS OF CORN DATA

VAR=2

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: ASINQ

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR F	R-SQUARE	C.V.
MODEL	10	3.49566819	0.34956682	26.43	0.0001	0.926386	35.1969
ERROR	21	0.27777750	0.01322750		ROOT MSE		ASINQ MEAN
CORRECTED TOTAL	31	3.77344568			0.11501087		0.32676389

SOURCE	DF	TYPE I SS	F VALUE	PR F	DF	TYPE III SS	F VALUE	PR F
REP	3	0.03933759	0.99	0.4161	3	0.03933759	0.99	0.4161
TRT	7	3.45633060	37.33	0.0001	7	3.45633060	37.33	0.0001

TABLE IX.

ANALYSIS OF VARIANCE: TRANSFORMED PERCENT STERILE FOR CORN VARIETY 3.

SPLIT PLOT ANALYSIS OF CORN DATA

VAR=3

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: ASINQ

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE	C.V.
MODEL	10	0	0	99999.99	0.0	0.000000	99999.9999
ERROR	21	0	0		ROOT MSE		ASINQ MEAN
CORRECTED TOTAL	31	0			0		0

SOURCE	DF	TYPE I SS	F VALUE	PR > F	DF	TYPE III SS	F VALUE	PR > F
REP	3	0	.	.	3	0	.	.
TRT	7	0	.	.	7	0	.	.

TABLE X.

ANALYSIS OF VARIANCE: TRANSFORMED PERCENT STERILE FOR CORN VARIETY 4.

SPLIT PLOT ANALYSIS OF CORN DATA

VAR=4

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: ASINQ

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR F	R-SQUARE	C.V.
MODEL	10	1.74120328	0.17412033	69.04	0.0001	0.970480	21.8082
ERROR	21	0.05296430	0.00252211		ROOT MSE		ASINQ MEAN
CORRECTED TOTAL	31	1.79416758			0.05022061		0.23028288

SOURCE	DF	TYPE I SS	F VALUE	PR F	DF	TYPE III SS	F VALUE	PR F
REP	3	0.01968844	2.60	0.0790	3	0.01968844	2.60	0.0790
TRT	7	1.72151484	97.51	0.0001	7	1.72151484	97.51	0.0001

Tables XI, XII, XIII and XIV contain the Duncan's multiple range test results for the comparisons of pairs of the sterility treatment means for corn varieties 1, 2, 3, and 4, respectively. Again, all treatment means specified as belonging to the same group, are not significantly different from one another, i.e., the percentage of plants that become sterile in each group of corn plants treated alike, do not differ between groups. Thus, means of treatments with sources 1, 2, 3, and 4 AMS/vector (B1, B6, B4, and B5, respectively) are significantly different from those of treatment with corn extract (no AMS/vector), buffer only, alfalfa extract (no AMS/vector), and untreated plants (B2, B3, B8, and B7, respectively), in varieties 1, 2 and 4. No male sterility was observed in variety 3.

TABLE XI.

DUNCAN'S MULTIPLE RANGE TEST FOR VARIABLE: TRANSFORMED PERCENT STERILE FOR CORN VARIETY 1.

ALPHA=.05 DF=21 MSE=.0031057

NUMBER OF MEANS	2	3	4	5	6	7	8
CRITICAL RANGE	0.0818478	0.0859649	0.0888102	0.0904927	0.0918322	0.0928782	0.0936988

DUNCAN GROUPING*	MEAN	N	TREATMENT
A	0.43940	4	B6
A A	0.42559	4	B1
A A	0.41104	4	B4
B	0.32070	4	B5
C	0.00000	4	B2
C C	0.00000	4	B3
C C	0.00000	4	B7
C C	0.00000	4	B8

*MEANS WITH THE SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT.

TABLE XII.

DUNCAN'S MULTIPLE RANGE TEST FOR VARIABLE: TRANSFORMED PERCENT STERILE FOR CORN VARIETY 2.

NUMBER OF MEANS	2	3	4	5	6	7	8
CRITICAL RANGE	0.168913	0.17741	0.183282	0.186754	0.189519	0.191677	0.193371

DUNCAN GROUPING*	MEAN	N	TREATMENT
A	0.70608	4	B1
A A	0.67560	4	B4
A A	0.66010	4	B5
A A	0.57233	4	B6
B	0.00000	4	B2
B B	0.00000	4	B3
B B	0.00000	4	B7
B B	0.00000	4	B8

*MEANS WITH THE SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT.

TABLE XIII.

DUNCAN'S MULTIPLE RANGE TEST FOR VARIABLE: TRANSFORMED PERCENT STERILE FOR CORN VARIETY 3.

SPLIT PLOT ANALYSIS OF CORN DATA
VAR=3
GENERAL LINEAR MODELS PROCEDURE

DUNCAN'S MULTIPLE RANGE TEST FOR VARIABLE: ASINQ
NOTE: THIS TEST CONTROLS THE TYPE I COMPARISONWISE ERROR RATE,
NOT THE EXPERIMENTWISE ERROR RATE

ALPHA=.05 DF=21 MSE=0

NUMBER OF MEANS	2	3	4	5	6	7	8
CRITICAL RANGE	0	0	0	0	0	0	0

DUNCAN GROUPING*	MEAN	N	TREATMENT
A	0	4	B1
A A	0	4	B2
A A	0	4	B3
A A	0	4	B4
A A	0	4	B5
A A	0	4	B6
A A	0	4	B7
A A	0	4	B8

*MEANS WITH THE SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT.

TABLE XIV.

DUNCAN'S MULTIPLE RANGE TEST FOR VARIABLE: TRANSFORMED PERCENT STERILE FOR CORN VARIETY 4.

ALPHA=.05 DF=21 MSA=.0025221

NUMBER OF MEANS	2	3	4	5	6	7	8
CRITICAL RANGE	0.0737576	0.0774678	0.0800318	0.081548	0.0827552	0.0836977	0.0844372

DUNCAN GROUPING*	MEAN	N	TREATMENT
A	0.49016	4	B4
A A	0.48661	4	B1
A A	0.47171	4	B5
B	0.39379	4	B6
C	0.00000	4	B2
C C	0.00000	4	B3
C C	0.00000	4	B7
C C	0.00000	4	B8

*MEANS WITH THE SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT.

6.10.2.1.2. ANALYSIS OF PLANT HEIGHT (TABLES XV THROUGH XVIII)

The analysis of variance for plant height indicated that there were significant differenc s among treatments (P less than 0.0106). This means that the probability that all of the sterility (including control) treatments had no effect on plant height is about 1 percent or 11 in 1,000. In other words, there is strong evidence that the sterility treatments affected the plant height (Tables XV, XVI).

TABLE XV.

AVERAGE PLANT HEIGHT RESPONSES FOR EACH STERILITY TREATMENT (1 - 8) FOR CORN VARIETIES 1 - 4.

	TREATMENT							
	B1	B2	B3	B4	B5	B6	B7	B8
	Source 1 AMS/Vector	Corn Extract (No AMS/Vector)	Buffer	Source 3 AMS/Vector	Source 4 AMS/Vector	Source 2 AMS/Vector	Untreated	Alfalfa Extract, (No AMS/Vector)
VARIETY	PLANT HEIGHT							
1	61.53	65.18	67.93	66.68	69.90	65.08	65.23	70.20
2	67.13	70.38	71.20	70.83	74.18	68.13	70.63	72.43
3	53.15	52.67	57.45	57.65	57.25	53.53	53.87	57.00
4	72.33	76.00	78.08	78.28	80.78	74.75	76.10	80.03

TABLE XVI.

ANALYSIS OF VARIANCE: PLANT HEIGHT.

REPEATED MEASURES ANALYSIS OF CORN DATA

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: PLNTHT

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR F	R-SQUARE	C.V.
MODEL	55	10279.30242188	186.89640767	21.91	0.0001	0.943622	4.3359
ERROR	72	614.14812500	8.52983507		ROOT MSE		PLNTHT MEAN
CORRECTED TOTAL	127	10893.45054688			2.92058814		67.35859375

SOURCE	DF	TYPE I SS	F VALUE	PR F	DF	TYPE III SS	F VALUE	PR F
REP	3	1017.80335938	39.77	0.0001	3	1017.80335938	39.77	0.0001
TRT	7	645.79867188	10.82	0.0001	7	645.79867188	10.82	0.0001
REP*TRT	21	539.01601562	3.01	0.0003	21	539.01601562	3.01	0.0003
VAR	3	7999.83648438	312.62	0.0	3	7999.83648438	312.62	0.0
TRT*VAR	21	76.84789063	0.43	0.9840	21	76.84789063	0.43	0.9840

TESTS OF HYPOTHESES USING THE TYPE III MS FOR REP*TRT AS AN ERROR TERM

SOURCE	DF	TYPE III SS	F VALUE	PR F
REP	3	1017.80335938	13.22	0.0001
TRT	7	645.79867188	3.59	0.0106

In order to determine which treatm nts were significantly different from one another, the multiple comparison procedure known as Duncan's multiple range test was performed. This statistical analysis allowed us to compare pairs of means without increasing the probability of making the mistake of declaring some comparisons to be significantly different, when in fact they were not.

As indicated in Table XVII, all means within the specific grouping are not significantly different from one another, while any two means from different groups can be declared as significantly different with only a 1 in 20 chance of being wrong. Thus, means of treatments with buffer alone, sources 3 and 4 AMS/vector, and alfalfa extract (no AMS/vector) (B3, B4, B5, and B8, respectively) were significantly different from those of treatments with sources 1 and 2 AMS/vector, corn extract (no AMS/vector), and untreated plants (B1, B6, B2, and B7, respectively).

TABLE XVII.

DUNCAN'S MULTIPLE RANGE TEST FOR VARIABLE: PLANT HEIGHT.

ALPHA=.05 DF=72 MSE=8.52984

NUMBER OF MEANS	2	3	4	5	6	7	8
CRITICAL RANGE	2.06032	2.16646	2.23575	2.28815	2.32836	2.36272	2.39075

DUNCAN GROUPING*	MEAN	N	TREATMENT
A	70.525	16	B5
A A	69.913	16	B8
A A	68.663	16	B3
A A	68.356	16	B4
B A	66.456	16	B7
B C	66.056	16	B2
C C	65.369	16	B6
D C	63.531	16	B1
D			

*MEANS WITH THE SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT.

The overall analysis of varianc also showed significant differences (P less than 0.0001) among varieties of corn plants (Table XVI, supra). The probability of erroneously concluding that the plant height response is different for each corn variety is much less than 0.01 percent or 1 in 10,000. In addition, the differences among treatments is the same for each variety of corn.

Table XVIII contains the results from a Duncan's multiple range test among the means of corn varieties (i.e., averaged across treatments).

TABLE XVIII.

DUNCAN'S MULTIPLE RANGE TEST FOR VARIABLE: PLANT HEIGHT.

ALPHA=.05 DF=72 MSE=8.52984

NUMBER OF MEANS	2	3	4
CRITICAL RANGE	1.45687	1.53192	1.58091

DUNCAN GROUPING*	MEAN	N	VARIETY
A	70.041	32	4
B	70.609	32	2
C	66.463	32	1
D	55.322	32	3

*MEANS WITH THE SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT.

Table XVIII, as with all subsequent tables of Duncan's multiple range test results, can be interpreted as explained for Table XVII above. Table XVIII indicates that all comparisons of plant height between any two varieties were significantly (P less than 0.05) different. The probability of erroneously concluding that the average plant height for one variety differs significantly from any other is less than or equal to 5 percent or 1 in 20.

6.10.2.1.3. ANALYSIS OF EAR HEIGHT (TABLES XIX THROUGH XXII)

The analysis of variance results for ear height show that there were no statistically significant differences among the sterility treatment means. Ear height appeared to be unaffected by any of the eight treatments used in the experiment (Tables XIX, XX).

TABLE XIX.

AVERAGE EAR HEIGHT RESPONSES FOR EACH STERILITY TREATMENT (1 - 8) FOR CORN VARIETIES 1 - 4.

	TREATMENT							
	B1	B2	B3	B4	B5	B6	B7	B8
	Source 1 AMS/Vector	Corn Extract (No AMS/Vector)	Buffer	Source 3 AMS/Vector	Source 4 AMS/Vector	Source 2 AMS/Vector	Untreated	Alfalfa Extract, (No AMS/Vector
VARIETY:	EAR HEIGHT							
1	25.18	27.00	27.55	26.95	27.08	25.60	25.30	28.58
2	29.37	30.25	29.15	32.60	34.80	29.90	29.23	31.00
3	18.50	19.38	21.75	22.48	20.50	17.75	18.87	22.70
4	25.68	28.73	29.18	28.37	34.37	28.43	28.75	29.37

TABLE XX.

ANALYSIS OF VARIANCE: EAR HEIGHT.

REPEATED MEASURES ANALYSIS OF CORN DATA

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: EARHT

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR F	R-SQUARE	C.V.
MODEL	55	3131.75742187	56.94104403	9.83	0.0001	0.882451	9.0161
ERROR	72	417.17187500	5.79405382		ROOT MSE		EARHT MEAN
CORRECTED TOTAL	127	3548.92929687			2.40708409		26.69765625

SOURCE	DF	TYPE I SS	F VALUE	PR F	DF	TYPE III SS	F VALUE	PR F
REP	3	225.27460937	12.96	0.0001	3	225.27460937	12.96	0.0001
TRT	7	251.36117187	6.20	0.0001	7	251.36117188	6.20	0.0001
REP*TRT	21	435.85601562	3.58	0.0001	21	435.85601563	3.58	0.0001
VAR	3	2055.63023438	118.26	0.0001	3	2055.63023437	118.26	0.0001
TRT*VAR	21	163.63539063	1.34	0.1773	21	163.63539063	1.34	0.1773

TESTS OF HYPOTHESES USING THE TYPE III MS FOR REP*TRT AS AN ERROR TERM

SOURCE	DF	TYPE III SS	F VALUE	PR F
REP	3	225.27460937	3.62	0.0300
TRT	7	251.36117188	1.73	0.1560

Even without a strong treatment effect, a Duncan's multiple range test can be us ful in uncov ring the patterns and relative magnitude of th diff renc s among treatment means. Table XXI pr sents th results of the Duncan's multiple range test among treatments.

TABLE XXI.

DUNCAN'S MULTIPLE RANGE TEST FOR VARIABLE: EAR HEIGHT TREATMENT MEANS.

ALPHA=.05 DF=72 MSE=5.79405

NUMBER OF MEANS	2	3	4	5	6	7	8
CRITICAL RANGE	1.69807	1.78555	1.84265	1.88419	1.91898	1.9473	1.9704

DUNCAN GROUPING*			MEAN	N	TREATMENT
		A	29.1875	16	B5
		A			
	B	A	27.9125	16	B8
	B	A			
	B	A	27.6000	16	B4
	B				
	B	C	26.9062	16	B3
	B	C			
	B	C	D 26.3375	16	B2
		C	D		
		C	D 25.5375	16	B7
		C	D		
		C	D 25.4187	16	B6
			D		
			D 24.6813	16	B1

*MEANS WITH THE SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT.

Ear height appeared to vary with the variety of corn plant. The analysis of variance results show that the probability of erroneously concluding that ear height varies significantly among varieties of corn plants was less than 0.01 percent or 1 in 10,000.

Table XXII contains the Duncan's multiple range test results for the corn plant variety means.

TABLE XXII.

DUNCAN'S MULTIPLE RANGE TEST FOR VARIABLE: EAR HEIGHT VARIETY MEANS.

ALPHA=.05 DF=72 MSE=5.79405

NUMBER OF MEANS	2	3	4
CRITICAL RANGE	1.20072	1.26258	1.30295

DUNCAN GROUPING*	MEAN	N	VAR
A	30.7875	32	2
B	29.1094	32	4
C	26.6531	32	1
D	20.2406	32	3

*MEANS WITH THE SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT.

Table XXII shows that each corn variety was significantly (P less than 0.05) different from each of the other three varieties. The probability that this conclusion is wrong is less than or equal to 5 percent or 1 in 20.

6.10.2.1.4. ANALYSIS OF DAYS TO SILKING (TABLES XXIII THROUGH XXVI)

The analysis of variance results indicate that none of the treatments have any effect on days to silking, but there is a variety effect. The days to silking differed significantly (P less than 0.0001) from one variety of corn plant to another (Tables XXIII, XXIV).

TABLE XXIII.

AVERAGE OF DAYS TO SILK RESPONSES FOR EACH STERILITY TREATMENT (1 - 8) FOR CORN PLANT VARIETIES 1-4.

	TREATMENT							
	B1	B2	B3	B4	B5	B6	B7	B8
VARIETY:	Source 1 AMS/Vector	Corn Extract (No AMS/Vector)	Buffer	Source 3 AMS/Vector	Source 4 AMS/Vector	Source 2 AMS/Vector	Untreated	Alfalfa Extract, (No AMS/Vector)
	DAYS TO SILK							
1	70.75	70.00	70.00	70.00	70.00	70.00	70.00	70.00
2	75.00	75.00	75.00	75.00	75.00	75.00	75.00	75.00
3	80.00	80.00	80.00	80.00	80.00	80.00	80.00	80.00
4	73.00	73.00	73.00	73.00	73.00	73.00	73.00	73.00

TABLE XXIV.

ANALYSIS OF VARIANCE: DAYS TO SILK.

REPEATED MEASURES ANALYSIS OF CORN DATA

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: SILKD

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR F	R-SQUARE	C.V.
MODEL	55	1672.86718750	30.41576705	432.58	0.0	0.996983	0.3558
ERROR	72	5.06250000	0.07031250		ROOT MSE		SILKD MEAN
CORRECTED TOTAL	127	1677.92968750			0.26516504		74.52343750

SOURCE	DF	TYPE I SS	F VALUE	PR F	DF	TYPE III SS	F VALUE	PR F
REP	3	0.21093750	1.00	0.3979	3	0.21093750	1.00	0.3979
TRT	7	0.49218750	1.00	0.4385	7	0.49218750	1.00	0.4385
REP*TRT	21	1.47656250	1.00	0.4743	21	1.47656250	1.00	0.4743
VAR	3	1669.21093750	7913.30	0.0	3	1669.21093750	7913.30	0.0
TRT*VAR	21	1.47656250	1.00	0.4743	21	1.47656250	1.00	0.4743

TESTS OF HYPOTHESES USING THE TYPE III MS FOR REP*TRT AS AN ERROR TERM

SOURCE	DF	TYPE III SS	F VALUE	PR F
REP	3	0.21093750	1.00	0.4123
TRT	7	0.49218750	1.00	0.4586

Duncan's multiple range test showed no significant differences between means of treatments (Table XXV).

TABLE XXV.

DUNCAN'S MULTIPLE RANGE TEST: TREATMENT MEANS FOR VARIABLE, DAYS TO SILK.

RELATED MEASURES ANALYSIS OF CORN DATA

GENERAL LINEAR MODELS PROCEDURE

DUNCAN'S MULTIPLE RANGE TEST FOR VARIABLE: SILKD
NOTE: THIS TEST CONTROLS THE TYPE I COMPARISONWISE ERROR RATE,
NOT THE EXPERIMENTWISE ERROR RATE

ALPHA=.05 DF=72 MSE=.0703125

NUMBER OF MEANS	2	3	4	5	6	7	8
CRITICAL RANGE	0.18706	0.196697	0.202987	0.207563	0.211395	0.214516	0.21706

DUNCAN GROUPING*	MEAN	N	TREATMENT
A	74.68750	16	B1
A A	74.50000	16	B2
A A	74.50000	16	B3
A A	74.50000	16	B4
A A	74.50000	16	B5
A A	74.50000	16	B6
A A	74.50000	16	B7
A A	74.50000	16	B8

*MEANS WITH THE SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT.

Table XXVI contains the results of the Duncan's multiple range test for comparisons of corn plant variety means for days to silking variable. All possible comparisons of the two varieties are significantly (P less than 0.05) different. The probability that this statement is in error is less than or equal to 5 percent or 1 in 20.

TABLE XXVI.

DUNCAN'S MULTIPLE RANGE TEST FOR VARIABLE: DAYS TO SILK.

ALPHA=.05 DF=72 MSE=.0703125

NUMBER OF MEANS	2	3	4
CRITICAL RANGE	0.132271	0.139086	0.143534

DUNCAN GROUPING*	MEAN	N	VAR
A	80.00000	32	3
B	75.00000	32	2
C	73.00000	32	4
D	70.09375	32	1

*MEANS WITH THE SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT.

6.10.2.1.5. SUMMARY OF STATISTICAL SIGNIFICANCE OF TREATMENT DIFFERENCES

Analysis of variance showed significant treatment differences at P less than 0.0001, for the pollen sterility character. Treatments with sources 1, 2, 3, and 4 AMS/vectors (B1, B6, B4, and B5, respectively) showed male sterility, while treatments with corn extract (no AMS/vector), buffer, alfalfa extract (no AMS/vector), and untreated plants (B2, B3, B8, and B7, respectively) did not. A strong variety effect was also apparent. In varieties 1, 2, and 4, there was strong evidence (P less than 0.0001) that corn plant sterility was effected by the treatments used. There was no statistically significant evidence of a treatment effect in variety 3. Since the analysis of variance showed a strong variety effect, comparison of treatment means were conducted for the three varieties i.e., 1, 2, and 4, which showed sterility. Duncan's multiple range test further revealed that treatments with corn extract (no AMS/vector), buffer, alfalfa extract (no AMS/vector), and untreated plants (B2, B3, B8, and B7, respectively) were significantly different from those with sources 1, 2, 3, and 4 AMS/vectors (B1, B6, B4, and B5, respectively) for all the varieties. In variety 1, means for treatment with source 4 AMS/vector (B5) were different from the rest of the treatment means, while in variety 4, means of treatment with source 2 AMS/vector (B6) were different from the rest of the treatment means.

Differences among treatments were apparent for the "plant height" character, both from the analysis of variance data and the Duncan's multiple range test. Significant differences (P less than 0.05) were also revealed for plant height character among varieties using Duncan's multiple range test.

Ear height was not affected by any of the eight treatments, but appeared to vary significantly with the

variety of corn plant. Means of ear height of all four varieti s were significantly differ nt from each other.

There was no significant differenc s between treatments for the "days to silking" variable. Both analysis of variance (P less than 0.0001) and Duncan's multiple range test P less than 0.05) revealed a significant varietal difference.

6.10.2.2. DESCRIPTION OF FEATURES OF STERILITY

Treatments with sources 1, 2, 3, and 4 AMS/vector (B1, B6, B4 and B5, respectively) showed plants without any dehisced pollen across all replicates. In tassels which showed no dehisced pollen, the anthers were covered by the glumes. In tassels which showed pollen when shaken on black paper, the anthers were out of the glumes and were easily seen. Plants showing no dehisced pollen were often seen clustered together, a position effect that was observed among all the treatments showing this feature and across the four replicates.

Microscopic examinations of anthers from tassels that have dehisced pollen were made for all four varieties across treatments and replicates. In all four varieties, the pollen in such tassels was characteristically round and uniform with a dense cytoplasm and was stainable with acetocarmine (Fig. 3). However, anthers from tassels that showed no dehisced pollen, in varieties 1 and 2, showed no dehiscence, and the abundance of abnormal, irregularly shaped, empty-looking pollen was clearly visible through the anther wall under a microscope (Fig. 4). The pollen from these anthers could be released only when considerable pressure was put on the anthers by pressing the coverslip (Figs. 5A, 5B). In variety 4, anthers from tassels that had no dehisced pollen did not form pollen grains, with the exception of a few tassels (Table XXVII). Even after

crushing the anthers, no sporogenous tissue was apparent (Fig. 6).

TABLE XXVII.

MICROSCOPIC RATINGS OF ANTHERS FROM TASSELS THAT WERE RATED FOR "NO DEHISCED POLLEN" IN THE VISUAL RATINGS.

Treatment	Variety 1			Variety 2			Variety 4		
	Total tassels examined	No pollen, no dehiscence	Abnormal pollen, no dehiscence	Total tassels examined	No pollen, no dehiscence	Abnormal pollen, no dehiscence	Total tassels examined	No pollen, no dehiscenc	Abnormal pollen, no dehiscence
B1	15	0	15* (100%)**	45	2 (4%)	43 (96%)	22	22 (100%)	0
B4	17	0	17 (100%)	48	4 (8%)	44 (92%)	22	22 (100%)	0
B5	14	0	14 (100%)	43	0	43 (100%)	20	16 (80%)	4 (20%)
B6	21	0	21 (100%)	33	1 (3%)	32 (97%)	16	13 (81%)	3 (19%)

*Tassels showing this feature
**Percentage of total tassels.

6.11. GROWTH ROOM TEST OF AMS/VECTOR TREATMENT ON SOYBEAN PLANTS

The examples described herein demonstrate the induction of male sterility, mediated by the AMS/vector, in soybeans.

Four treatments containing AMS/vector and four control treatments were applied to soybeans, four weeks after emergence (before flowering), to test whether the AMS/vector induces male sterility in soybeans. Microscopic examination of anthers and pollen were made to assess the effects of AMS/vector treatment. Plant height, number of flowering nodes, and number of pods were determined for each plant after completing the pollen examination, in order to see if there were any other effects of the treatment applications such as plant growth stimulation. The statistical significance of treatment effects was determined using analysis of variance and Duncan's multiple range test.

Male sterile plants were observed in treatments with sources 1, 2, 3, and 4 AMS/vector. Treatment with soybean extract (no AMS/vector) resulted in one male sterile plant out of 41 plants examined. Treatments with buffer alone, alfalfa extract (no AMS/vector), or no treatment, produced no male sterile plants. Statistical analysis was performed across all treatments and replicates using a microscopic rating of 1-7 for pollen sterility. Analysis of variance indicated that the treatment differences were highly significant (P less than 0.0001) for the microscopic rating of pollen sterility. Duncan's multiple range test revealed that the treatment means with sources 1, 2, 3, and 4 AMS/vector were significantly different from those of treatment with buffer alone, alfalfa extract (no AMS/vector), or no treatment. Analysis of variance did not show any treatment differences for plant height, flowering nodes/plant, and number of pods/plant. When Duncan's

multiple range t st was used, means of treatments with source 1 AMS/vector and with alfalfa (no AMS/vector) differed significantly from the m ans of treatments with source 2 AMS/vector and with no treatment, for plant height variable, and the mean of treatment with source 3 AMS/vector differed from treatment with alfalfa (no AMS/vector) for number of pods/plant. Duncan's multiple range test did not reveal any treatment differences in flowering nodes/plant.

Three distinct patterns were observed during the microscopic examination of flowers: one in which the flowers had normal looking anthers, abundant pollen that was regular in shape and size and was stainable with acetocarmine; a second in which the anthers were normal looking, but had inside pollen that was a mix of abnormal and normal pollen; and a third in which there were no pollen grains in an apparently normal looking anther.

6.11.1. MATERIALS AND METHODS

6.11.1.1. SOYBEAN SEED SOURCE

Seeds of soybean used in the experiment described herein were Williams-82 variety. The seeds were shipped overnight to the field test site. Seed was stored in a cold room at 38°F until planting. The seed was received in eight batches in seed envelopes designated T1-T8.

6.11.1.2. COLLECTION AND SHIPMENT OF TEST MATERIALS

Alfalfa material was cut fresh from the field and immersed in liquid nitrogen. The liquid nitrogen frozen material was shipped overnight in dry ice to the field test site. Four of these materials (from four male sterile alfalfa lines, PI Nos. 221469, 172429, 223386, and 243223) contained AMS/vector, and one was an alfalfa control (an isogenic non-sterile line). This material arrived in sealed plastic bags, precoded as T1, T2, T3, T4, and T5. A record

was kept of the sources for treatments and controls and their corresponding T numbers. Personnel who did th field test were aware only of the 'T' designations, not the nature of the treatments in each case. Those who performed the field test were therefore "blind" to the treatments.

6.11.1.3. GROWTH SYSTEM AND CONDITIONS FOR PLANT GROWTH

A sterile, plant growth assembly was used for growing soybeans. The plants were fed a nutrient solution containing macro and micronutrients as follows: 1.08 g $CaHPO_4$, 0.2 g K_2HPO_4, 0.2 g $MgSO_4$, 0.2 g NaCl, 0.16 g $FeCl_3$, 1000 ml water. One ml of trace elements Bo - 0.05%; Mn - 0.05%; Zn - 0.005%; Mo - 0.005%; and Cu - 0.002% were added. This nutrient solution was used at one-tenth strength, and supplemented with KNO_3 (0.05%) as the nitrogen source.

The controlled environment growth room for this experiment was set 14 hour day/10 hour night cycle, with a constant temperature regime of 25°C, and a light intensity at the plant canopy of 340 umol $m^{-2}s^{-1}$.

6.11.1.4. PLANTING AND GERMINATION

The seeds from each packet (T1-T8) were emptied into sterilized jars, and surface sterilized by rinsing momentarily with 50 ml of 95% ethanol and then treating for 1.5 minutes with acidified mercuric chloride solution (0.2% $HgCl_2$, 0.5% concentrated HCl in water). The mercuric chloride solution was decanted and the seeds rinsed 10 times with sterile distilled water. After the final rinse, the seeds were left to imbibe in sterile distilled water for one hour before planting. Three seeds were planted per pot at 1.5 to 2 cm depth in the soil. The surface of the soil was covered with one inch of sterile aquarium gravel after planting to prevent bacterial and fungal contamination. The entire assembly was wrapped with brown paper to shield the soil and root system from light. Pots were labeled (T1-T8),

and transferred to the growth room. A total of 24 pots were planted with each of the eight batches of seed.

Germination data w re recorded six days after planting. Germination p rcentag s not d for each batch of seed were as follows: T1 - 44; T2 - 42; T3 - 35; T4 - 54; T5 - 47; T6 -39; T7 - 33; T8 - 32. Germination was lower than is usual (usual being greater than 90%), so all seedlings in the pots were retained and no thinning was done.

Additional seeds of cultivar Williams-82 (from DeWine Seed Company, Yellow Springs, Ohio) were sterilized and planted into pots with less than three seedlings, using the same procedure as above. Seeds were planted to achieve a minimum of three seedlings per pot. Seedlings corresponding to the original seed batches were labeled to distinguish them from the additional group of Williams-82 seed. Only seeds of Williams-82 of the additional group were thinned whenever the seedlings exceeded three per pot.

Subsequently, a second set of pots was planted with soybean seeds, as eight batches of seed from packets labelled T1-T8 containing 20 seeds each. These seeds were kept in a cold room at 38°F until planting. Two seeds were planted in each of 80 pots, 10 pots per packet of seeds. The plant growth system and planting procedures were similar to the first planting, except that the seeds in the second planting were not surface sterilized. The seeds in the second planting were not surface sterilized because there were fine cracks in the seed coat from shipping damage, and the sterilization procedure was penetrating the seed through these fine cracks and reducing viability and germination. Germination percentages of the seed in the second planting were as follows: T1 - 80; T2 - 100; T3 - 70; T4 - 80; T5 - 60; T6 - 100; T7 - 90; T8 - 65.

After germination of the second planting, pots from the first planting in which none of the original seeds had germinated were discarded. The number of pots discarded were

as follows: T1 - 0; T2 - 6; T3 - 7; T4 - 1; T5 - 2; T6 - 4; T7 - 5; T8 - 3.

6.11.1.5. EXPERIMENTAL DESIGN AND TREATMENTS

The experimental design was a split-plot with replications as the whole plot and treatments as the split. The split-plot design helps reduce error by keeping treatment blocks together. There is still randomization of plants within each treatment, and of treatments within each replication. (A completely randomized design would not separate treatments into blocks).

There were six replications of eight treatments. The eight treatments (described in Table XXVIII) included four materials which contained AMS/vector from different alfalfa genotypes as sources, and various controls.

TABLE XXVIII.

SOYBEAN PROJECT TREATMENT AND CODES

Treatment	Code 1 Field Site Code	Code 2
Source 1, AMS/vector (U.S.D.A. PI No. 221469)	B6	T1
Untreated	B5	T2
Soybean extract, no AMS/vector	B7	T3
Source 2, AMS/vector (U.S.D.A. PI No. 172429)	B8	T4
Source 3, AMS/vector (U.S.D.A. PI No. 223386)	B2	T5
Alfalfa extract, no AMS/vector	B3	T6
Buffer only, no plant extract	B1	T7
Source 4, AMS/vector (U.S.D.A. PI No. 243223)	B4	T8

6.11.1.6. PREPARATION AND APPLICATION OF AMS/VECTOR TREATMENTS AND CONTROL TREATMENT

6.11.1.6.1. TREATMENTS AND THEIR SOURCES

The alfalfa material received and stored frozen, was the source material for the four AMS/vector treatments and the alfalfa extract control. The source material for soybean extract was var. Williams grown at the field site. The other two control treatments involved application of buffer (0.067 M KH_2PO_4, pH 6.9) only, or no material applied

("untreated") b yond the C lite application common to all treatments.

6.11.1.6.2. EXTRACTION PROCEDURE

Phosphate buffer (KH_2PO_4, 0.67 M, pH 6.9) was prepared three days before the extraction of plant material and was kept stored at 11°C. All of the extraction procedures were performed while wearing disposable surgical gloves. A new pair of gloves was used for each treatment to avoid cross-contamination.

The frozen alfalfa plant material was taken out of the freezer and weighed. For each extract, 160 g of material was macerated in 800 ml of KH_2PO_4 buffer (0.067 M, pH 6.9, 11°C), in a Waring heavy duty blender for 2-3 minutes. The homogenate was filtered through four layers of sterile cheese cloth to remove the plant debris. The filtrate was collected in sterilized 250 ml centrifuge bottles, and centrifuged at 2,000 rpm for 5 minutes using a GSA rotor in a refrigerated Sorvall centrifuge. The supernatant was decanted into sterile flasks, labeled, and stored at 38°F until used for spraying. The resultant supernatant constituted the extract for spraying the soybean plants.

Soybean extract was prepared in the same manner, from soybean plants grown at the field site.

6.11.1.6.3. APPLICATION OF EXTRACTS

Celite (diatomaceous earth, grade III, Sigma Chemicals Cat. No. D5384) was used as an abrador. One hundred grams of Celite was added to 1000 ml of KH_2PO_4 buffer (0.067 M, pH 6.9, 11°C) in a one gallon garden tank sprayer. The Celite-buffer mix was vigorously shaken, to ensure a uniform dispersion of Celite in the buffer for spraying.

The soybean plants were sprayed at a stage when the fifth internode appeared but no floral primordia were visible to the eye. All plants were sprayed first with the Celite-

buffer mixtur . Then plants w re tak n out of the growth room and sprayed with one treatment (extract) at a time. The six tr atments involving plant xtracts and the buffer-only control were spray d using an aerosol spray unit (Sigma Chemicals Cat. No. S4885) and an aerosol propellant refill (Sigma Chemicals Cat. No. A4532). Each soybean plant was sprayed starting from the first node and proceeding up to the shoot tip. The soybean plants were rotated during application. Approximately 25 ml of plant extract (or buffer only) were applied to each plant. Plants in one control treatment (no buffer, no extract) had only Celite applied.

At the time of spraying, non-field site personnel noted the 'T' number on the pots and their corresponding treatment code. Personnel at the field site then relabeled the T1-T8 pots with randomly assigned designations B1-B8. The field site personnel retained the code relating 'B' numbers to 'T' numbers (see Table XXVIII). From this point onwards, the study became a "double-blind" test in that no one could become aware of the nature of each treatment without breaking the codes held by separate parties. After the pots were labeled with the field site codes, they were transferred to the growth room and randomized within four blocks (replications).

Extract preparation and spraying of soybeans in the second planting of the additional seed was performed in the same manner as the first planting. The pots were then coded with the corresponding field site number (B1-B8), using the same code established for the first planting. These pots were arranged in the growth room and regarded as replicates 5 and 6, with eight treatments in each replicate. All plants were staked with garden stakes to keep them upright.

6.11.1.7. COLLECTION OF THE DATA

Data collection included four parameters: pollen stainability, plant height (inches) at 120 days, number of

flowering nodes, and number of pods per plant. These parameters were assess d for each plant in all of the treatments. Representative microscopic fields depicting pollen assessment were photographed.

Data collection for pollen stainability began when flowers appeared at the second node. Three flowers were chosen at random from each plant for the pollen stainability rating. Stamens from the flowers were transferred to a glass slide using tweezers. One drop of acetocarmine stain was placed on the stamens and covered with a cover slip. The cover slip was tapped gently and the stamens were observed under the microscope and rated for proven stainability as follows:

Rating 1 = No pollen present.
Rating 3 = Less than 5% of the pollen present became stained.
Rating 5 = 5-95% of the pollen present became stained.
Rating 7 = 96-100% of the pollen present became stained.

Representative photographs of anthers and pollen qualifying for these ratings were taken as the rating proceeded. The microscopic rating for pollen stainability was completed 120 days after planting, and then plant height, number of flowering nodes, and number of pods were measured.

6.11.1.8. STATISTICAL ANALYSIS OF THE DATA

Data was analyzed as a randomized block design. The statistical program Statistical Analysis System (SAS) was used for the analysis. Analysis of variance (F statistic) was used to test for statistically significant differences among the eight treatments. Significance probabilities less than or equivalent to a P value of 0.01 are consid red strong

evidences in favor of a treatment effect. Mean separations were done using Duncan's new multiple range test. Treatment means were compared using critical range values.

6.11.2. RESULTS AND DISCUSSION

6.11.2.1. STATISTICAL ANALYSIS

One of eight (including four control) treatments was randomly assigned to one of eight groups of potted plants. Each group contained six pots with two plants in each pot. The treatments were sprayed onto each plant individually. This design was replicated six times.

The experimental design described above is the familiar randomized-block design in which the eight treatments comprise the treatment main effect and each replicate constitutes a block.

Table XXIX contains the overall means of each of the four response variables measured in this investigation, i.e., flower rating, plant height, number of flower nodes, and number of pods.

TABLE XXIX.

TREATMENT MEANS FOR RESPONSE VARIABLES

Treatment	Flower Rating	Plant Height	Number of Flower Nodes	Number of Seed Pods
B1	5.80503	52.8340	21.4528	11.1837
B2	5.01389	49.7479	22.6667	8.6977
B3	5.56209	59.9784	21.8824	12.9565
B4	4.91026	51.2077	21.5000	9.1628
B5	5.60819	48.4860	21.6491	12.2642
B6	4.57692	54.6000	23.6731	11.2000
B7	5.43902	49.9732	21.6585	9.8378
B8	4.54167	47.4958	21.9167	9.5745

The means of the response variables of flower rating, plant height, number of flower nodes, and number of pods for each combination of treatment and replicate are given in Tables XXX, XXXIII, XXXVI, and XXXIX, infra.

6.11.2.1.1. ANALYSIS OF POLLEN RATING (TABLES XXX through XXXII)

Three separate flowers were chosen from each plant and rated for pollen sterility. An average of these three ratings was calculated for each plant (Table XXX) and was used as the response variable for analysis.

TABLE XXX.

FLOWER RATING MEANS FOR EACH COMBINATION OF TREATMENT AND REPLICATE.

REPLICATION	TREATMENT							
	B1	B2	B3	B4	B5	B6	B7	B8
	Buffer only	Source 3 AMS/Vector	Alfalfa Extract, no AMS/Vector	Source 4 AMS/Vector	Untreated	Source 1 AMS/Vector	Soybean Extract, no AMS/Vector	Source 2 AMS/Vector
	FLOWER RATING							
1	5.89	5.93	5.74	4.94	5.73	4.39	5.33	5.08
2	6.00	5.30	5.76	5.48	5.38	4.75	5.44	4.50
3	5.81	4.67	5.67	4.33	5.60	5.44	4.44	4.53
4	5.37	5.07	5.67	5.37	5.30	4.67	5.92	4.06
5	6.00	5.00	5.53	4.11	6.00	4.21	5.95	5.83
6	5.80	4.17	5.24	4.48	5.44	3.67	5.33	4.00

The analysis of variance results (Table XXXI) indicated that th re was a highly significant treatment effect (P less than 0.0001).

TABLE XXXI.

ANALYSIS OF VARIANCE: FLOWER RATING.

RANDOMIZED BLOCK ANALYSIS OF SOYBEAN DATA

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: FRATING

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR F	R-SQUARE	C.V.
MODEL	47	149.71388344	3.18540178	2.00	0.0002	0.209530	24.3422
ERROR	354	564.80684624	1.59549957		ROOT MSE		FRATING MEAN
CORRECTED TOTAL	401	714.52072968			1.26313086		5.18905473

SOURCE	DF	TYPE I SS	F VALUE	PR F	DF	TYPE III SS	F VALUE	PR F
REP	5	8.27082938	1.04	0.3957	5	15.04480759	1.89	0.0961
TRT	7	89.02542528	7.97	0.0001	7	78.33462931	7.01	0.0001
REP*TRT	35	52.41762879	0.94	0.5719	35	52.41762879	0.94	0.5719

TESTS OF HYPOTHESES USING THE TYPE III MS FOR REP*TRT AS AN ERROR TERM

SOURCE	DF	TYPE III SS	F VALUE	PR F
REP	5	15.04480759	2.01	0.1015
TRT	7	78.33462931	7.47	0.0001

The probability of making an error in judgment by concluding that there is a treatment effect wh n, in fact, there is none, is 0.01 percent or about 1 in 10,000. This is strong evidence that the average flower rating is affected by the range of sterility treatments.

A Duncan's multiple range test was performed at the 0.05 level, to review the pattern or magnitude of the differences between pairs of treatment means. Table XXXII contains the results of the Duncan's multiple range test.

TABLE XXXII.

DUNCAN'S MULTIPLE RANGE TEST FOR VARIABLE: FLOWER RATING.

ALPHA=.05 DF=354 MSE=1.5955

CELL SIZES ARE NOT EQUAL.
HARMONIC MEAN OF CELL SIZES=49.8324

NUMBER OF MEANS	2	3	4	5	6	7	8
CRITICAL RANGE	0.502695	0.528615	0.545294	0.557823	0.568368	0.576988	0.584038

DUNCAN GROUPING*	MEAN	N	TREATMENT
A	5.8050	53	B1
A A	5.6082	57	B5
A A	5.5621	51	B3
A A	5.4390	41	B7
B A	5.0139	48	B2
B B C	4.9103	52	B4
C C	4.5769	52	B6
C C	4.5417	48	B8

*MEANS WITH THE SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT.

As indicated in Table XXXII, all means belonging to the same group are not significantly different at the 0.05 level, but comparisons of any two treatment means between groups can be considered significant. That is, the probability of making an error in judgment by concluding that, say, flower ratings for treatment 2 are significantly different than flower ratings for treatment 3 is less than or equal to 5 percent or about 1 in 20. Treatment means of sources 1, 2, 3, and 4 AMS/vector (B6, B8, B2, and B4, respectively) were significantly different from treatment means of buffer only, alfalfa extract (no AMS/vector), and untreated plants (B1, B3, and B5, respectively) (Table XXXII).

6.11.2.1.2. ANALYSIS OF PLANT HEIGHT (TABLES XXXIII THROUGH XXXV)

The analysis of variance results indicated that there was no significant treatment effect on the height of the soybean plants in the experiment (Tables XXXIII, XXXIV).

TABLE XXXIII.

PLANT HEIGHT MEANS FOR EACH COMBINATION TREATMENT AND REPLICATE.

REPLICATION	TREATMENT							
	B1	B2	B3	B4	B5	B6	B7	B8
	Buffer only	Source 3 AMS/Vector)	Alfalfa Extract, no AMS/Vector	Source 4 AMS/Vector	Untreated	Source 1 AMS/Vector	Soybean Extract, no AMS/Vector	Sourc 2 AMS/Vector
	PLANT HEIGHT							
1	56.06	57.96	57.90	47.72	64.58	56.01	47.10	59.38
2	58.85	58.07	63.19	56.64	54.50	54.73	57.40	44.74
3	58.74	47.12	63.05	64.70	46.90	56.83	59.28	45.51
4	58.31	49.53	90.94	43.03	41.80	60.10	47.56	42.28
5	41.15	44.59	42.50	41.27	41.84	47.84	45.31	49.08
6	44.22	39.53	46.51	48.72	43.23	52.70	45.72	48.03

TABLE XXXIV.

ANALYSIS OF VARIANCE: PLANT HEIGHT.

RANDOMIZED BLOCK ANALYSIS OF SOYBEAN DATA

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: PLNTHT

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR F	R-SQUARE	C.V.
MODEL	47	32695.35078091	695.64576130	3.86	0.0001	0.338829	25.8945
ERROR	354	63799.92105988	180.22576571		ROOT MSE		PLNTHT MEAN
CORRECTED TOTAL	401	96495.27184080			13.42481902		51.84427861

SOURCE	DF	TYPE I SS	F VALUE	PR F	DF	TYPE III SS	F VALUE	PR F
REP	5	8140.72211101	9.03	0.0001	5	7676.88729557	8.52	0.0001
TRT	7	6553.78476375	5.19	0.0001	7	5546.33526705	4.40	0.0001
REP*TRT	35	18000.84390615	2.85	0.0001	35	18000.84390615	2.85	0.0001

TESTS OF HYPOTHESES USING THE TYPE III MS FOR REP*TRT AS AN ERROR TERM

SOURCE	DF	TYPE III SS	F VALUE	PR F
REP	5	7676.88729557	2.99	0.0239
TRT	7	5546.33526705	1.54	0.1861

Th probability of erroneously concluding that there was a significant tr atment effect is about 18 percent (Table XXXIV).

Again, a Duncan's multiple range test was p rformed to assess the pattern and magnitude of the pairwise differences between treatment means. Table XXXV contains the results of this test, and indicated that 9 of the possible 28 different pairs of treatment means could be declared significantly different. The probability that this statement is incorrect is less than or equal to 5 percent or about 1 in 20. Thus the overall main effect for treatments on plant height did not appear to be significant.

TABLE XXXV.

DUNCAN'S MULTIPLE RANGE TEST FOR VARIABLE: PLANT HEIGHT.

ALPHA=.05 DF=354 MSE=180.226

CELL SIZES ARE NOT EQUAL.
HARMONIC MEAN OF CELL SIZES=49.8324

NUMBER OF MEANS	2	3	4	5	6	7	8
CRITICAL RANGE	5.34275	5.618235	5.79549	5.92866	6.04073	6.13233	6.20728

DUNCAN GROUPING*	MEAN	N	TREATMENT
A	59.978	51	B3
B	54.600	52	B6
B			
C B	52.834	53	B1
C B			
C B	51.208	52	B4
C B			
C B	49.973	41	B7
C B			
C B	49.748	48	B2
C			
C	48.486	57	B5
C			
C	47.496	48	B8

*MEANS WITH THE SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT.

6.11.2.1.3. ANALYSIS OF THE NUMBER OF FLOWERING NODES (TABLES XXXVI THROUGH XXXVIII)

The analysis of variance results indicated that ther was no evidence for a tr atment eff ct (Tables XXXVI, XXXVII).

TABLE XXXVI.

MEAN NUMBER OF FLOWER NODES FOR EACH COMBINATION OF TREATMENT AND REPLICATE.

REPLICATION	TREATMENT							
	B1	B2	B3	B4	B5	B6	B7	B8
	Buffer only	Source 3 AMS/Vector)	Alfalfa Extract, no AMS/Vector	Source 4 AMS/Vector	Untreated	Source 1 AMS/Vector	Soybean Extract, no AMS/Vector	Sourc 2 AMS/Vector
	FLOWER NODES							
1	22.44	28.80	20.00	19.58	26.20	21.08	16.50	22.75
2	19.25	22.44	18.57	19.00	19.00	22.25	17.00	16.25
3	24.67	18.17	23.12	24.75	20.60	24.44	31.50	29.00
4	23.11	22.80	20.25	18.00	18.44	23.62	19.25	17.08
5	20.00	26.50	26.40	22.67	22.83	26.09	28.00	24.50
6	19.10	21.00	23.36	27.33	21.44	26.00	19.17	24.50

TABLE XXXVII.

ANALYSIS OF VARIANCE: FLOWER NODES.

RANDOMIZED BLOCK ANALYSIS OF SOYBEAN DATA

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: FNODES

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR F	R-SQUARE	C.V.
MODEL	47	4863.83397945	103.48582935	1.92	0.0005	0.202871	33.3226
ERROR	354	19111.17099567	53.98635874		ROOT MSE		FNODES MEAN
CORRECTED TOTAL	401	23975.00497512			7.34754100		22.04975124

SOURCE	DF	TYPE I SS	F VALUE	PR F	DF	TYPE III SS	F VALUE	PR F
REP	5	1403.70252625	5.20	0.0001	5	1475.41938225	5.47	0.0001
TRT	7	191.28914577	0.51	0.8298	7	259.98006578	0.69	0.6823
REP*TRT	35	3268.84230744	1.73	0.0077	35	3268.84230744	1.73	0.0077

TESTS OF HYPOTHESES USING THE TYPE III MS FOR REP*TRT AS AN ERROR TERM

SOURCE	DF	TYPE III SS	F VALUE	PR F
REP	5	1475.41938225	3.16	0.0186
TRT	7	259.98006578	0.40	0.8972

The probability of making an error in judgment by concluding that the number of flower nodes was significantly affected by the treatments used in this study was nearly 90 percent (Table XXXVII).

A Duncan's multiple range test also showed no significant pairwise differences among treatment means (Table XXXVIII).

TABLE XXXVIII.

DUNCAN'S MULTIPLE RANGE TEST FOR VARIABLE: FLOWER NODES.

RANDOMIZED BLOCK ANALYSIS OF SOYBEAN DATA

GENERAL LINEAR MODELS PROCEDURE

DUNCAN'S MULTIPLE RANGE TEST FOR VARIABLE: FNODES

NOTE: THIS TEST CONTROLS THE TYPE I COMPARISONWISE ERROR RATE, NOT THE EXPERIMENTWISE ERROR RATE

ALPHA=.05 DF=354 MSE=53.9864

CELL SIZES ARE NOT EQUAL. HARMONIC MEAN OF CELL SIZES=49.8324

NUMBER OF MEANS	2	3	4	5	6	7	8
CRITICAL RANGE	2.92414	3.07492	3.17193	3.24482	3.30615	3.35629	3.39731

DUNCAN GROUPING*	MEAN	N	TREATMENT
A	23.673	52	B6
A A	22.667	48	B2
A A	21.917	48	B8
A A	21.882	51	B3
A A	21.659	41	B7
A A	21.649	57	B5
A A	21.500	52	B4
A A	21.453	53	B1

*MEANS WITH THE SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT

6.11.2.1.4. ANALYSIS OF THE NUMBER OF PODS (TABLES XXXIX THROUGH XLI)

Analysis of variance results showed no evidence that the number of pods per plant was significantly affected by any of the sterility treatments (Tables XXXIX, XL).

TABLE XXXIX.

MEAN NUMBER OF SEED PODS FOR EACH COMBINATION OF TREATMENT AND REPLICATE.

REPLICATION	TREATMENT							
	B1	B2	B3	B4	B5	B6	B7	B8
	Buffer only	Source 3 AMS/Vector)	Alfalfa Extract, no AMS/Vector	Source 4 AMS/Vector	Untreated	Source 1 AMS/Vector	Soybean Extract, no AMS/Vector	Source 2 AMS/Vector
	NUMBER OF PODS							
1	10.33	14.80	14.25	10.11	15.70	6.67	9.29	12.25
2	10.62	7.89	13.83	8.22	14.86	9.50	7.00	7.00
3	13.56	8.89	16.25	13.71	12.40	24.87	10.00	15.40
4	14.25	6.88	12.12	7.57	11.71	10.86	10.00	3.58
5	9.43	8.25	11.50	5.33	9.50	8.91	14.43	14.25
6	8.50	7.00	10.50	8.00	9.86	7.75	5.33	8.17

TABLE XL.

ANALYSIS OF VARIANCE: PODS.

RANDOMIZED BLOCK ANALYSIS OF SOYBEAN DATA

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: PODS

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR F	R-SQUARE	C.V.
MODEL	47	5023.77617479	106.88885478	1.63	0.0080	0.193476	75.6359
ERROR	320	20942.13686869	65.44417771		ROOT MSE		PODS MEAN
CORRECTED TOTAL	367	25965.91304348			8.08975758		10.69565217

SOURCE	DF	TYPE I SS	F VALUE	PR F	DF	TYPE III SS	F VALUE	PR F
REP	5	1379.83584045	4.22	0.0010	5	1348.45376309	4.12	0.0012
TRT	7	788.59635545	1.72	0.1032	7	724.46927394	1.58	0.1400
REP*TRT	35	2855.34397889	1.25	0.1668	35	2855.34397889	1.25	0.1668

TESTS OF HYPOTHESES USING THE TYPE III MS FOR REP*TRT AS AN ERROR TERM

SOURCE	DF	TYPE III SS	F VALUE	PR F
REP	5	1348.45376309	3.31	0.0150
TRT	7	724.46927394	1.27	0.2940

The probability of erroneously concluding that the number of pods was significantly affected by the sterility treatments is about 30 percent or 3 in 10 (Table XL).

A Duncan's multiple range t st indicated that only the differences between the means of treatment B2 (source 3 AMS/vector) and treatment B3 (alfalfa extract, no AMS/vector) can be declared significant, with only a 5 percent probability of being wrong (Table XLI).

TABLE XLI.

DUNCAN'S MULTIPLE RANGE TEST FOR VARIABLE: PODS.

RANDOMIZED BLOCK ANALYSIS OF SOYBEAN DATA
GENERAL LINEAR MODELS PROCEDURE

DUNCAN'S MULTIPLE RANGE TEST FOR VARIABLE: PODS

NOTE: THIS TEST CONTROLS THE TYPE I COMPARISONWISE ERROR RATE, NOT THE EXPERIMENTWISE ERROR RATE

ALPHA=.05 DF=320 MSE=65.4442

CELL SIZES ARE NOT EQUAL. HARMONIC MEAN OF CELL SIZES=45.4984

NUMBER OF MEANS	2	3	4	5	6	7	8
CRITICAL RANGE	3.36938	3.54311	3.6549	3.73888	3.80956	3.86732	3.91459

DUNCAN GROUPING*		MEAN	N	TREATMENT
	A	12.957	46	B3
	A			
B	A	12.264	53	B5
B	A			
B	A	11.200	50	B6
B	A			
B	A	11.184	49	B1
B	A			
B	A	9.838	37	B7
B	A			
B	A	9.574	47	B8
B	A			
B	A	9.163	43	B4
B				
B		8.698	43	B2

*MEANS WITH THE SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT.

6.11.2.1.5. SUMMARY OF STATISTICAL SIGNIFICANCE OF TREATMENT DIFFERENCES

Tr atm nt differences w re highly significant (P l ss than 0.0001) for th microscopic rating of pollen sterility based on the analysis of variance data. Duncan's multiple range test, performed to review the magnitude of differences between pairs of treatments, revealed that treatment means of sources 1, 2, 3, and 4 AMS/vector (B6, B8, B2, and B4, respectively) were significantly different from treatment means of buffer alone, alfalfa extract (no AMS/vector), and untreated plants (B1, B3, and B5, respectively).

Analysis of variance results indicated no significant treatment effects on the height of the soybean plant. However, Duncan's multiple range test revealed that means of treatments B6 (source 1 AMS/vector) and B3 (alfalfa extract, no AMS/vector) significantly differed from the means of treatments B8 (source 3 AMS/vector) and B5 (untreated).

There were no significant treatment effects on the number of flowering nodes/plant, either by analysis of variance or Duncan's multiple range test. There was also no evidence that the number of pods was significantly affected by any treatment from the analysis of variance test. However, Duncan's multiple range test revealed differences between means of treatments B2 (source 3 AMS/vector) and B3 (alfalfa extract, no AMS/vector).

6.11.2.2. DESCRIPTION OF FEATURES OF STERILITY

Male sterile plants were observed in treatments with sources 1, 2, 3, and 4 AMS/vector (B6, B8, B2, and B4, respectively). In treatment with soybean extract (no AMS/vector) (B7) only one out of 41 plants was male sterile. Treatments with buffer alone, alfalfa extract (no AMS/vector), and untreated plants (B1, B3, and B5, respectively) had no male sterile plants (Table XLII).

TABLE XLII.

SOYBEAN-OVERALL STERILITY PROFILE ACROSS SIX REPLICATES

Treatment	Plants Examined	Sterile* Plants	Fertile Plants
B1	53	0** (0%)	53 (100%)
B2	48	6 (12%)	42 (88%)
B3	51	0 (0%)	51 (100%)
B4	52	6 (12%)	46 (88%)
B5	57	0 (0%)	57 (100%)
B6	52	12 (23%)	40 (77%)
B7	41	1 (2%)	40 (98%)
B8	48	10 (21%)	38 (79%)

*Plants had flowers with predominantly 1 rating (no pollen) and a few where less than 5% of the pollen became stained (3 rating). Sterile plants did not have pods.

**Actual number of plants, with percentages in parenthesis.

Representative patterns of sterility observed during microscopic ratings of flowers from treated plants (Figs. 7-13) revealed three distinct patterns.

The first pattern showed flowers with anthers that had abundant pollen grains (Fig. 7) which were uniform in size and shape and were stained red with acetocarmine (Fig. 8). The stigmatic surface of such flowers had a mass of pollen grains attached to it (Fig. 9). Soybean is a self-pollinated species and Figures 7-9 show characteristic features that could be seen in a normal soybean flower.

The second pattern was characterized by flowers that had normal looking anthers, but the anther contents were a mix of non-stainable, abnormally shaped poll n grains and normal pollen (Fig. 10). The abnormal pollen was of

irregular shape, non-stainable and highly vacuolated (Fig. 11). The normal to abnormal ratio varied from flower to flower in this pattern.

The third pattern was characterized by flowers that had normal looking anthers, but the anthers lacked any pollen grains (Fig. 12). The stigma on such a flower lacked pollen and stigmatic hairs on its surface. Such anthers, even aft r being crushed, did not reveal any pollen grains inside them (Fig. 13).

6.11.2.3. ADDENDUM TO STATISTICAL ANALYSIS

A randomized block design was used as the basis for the analysis of variance for flower rating, plant height, number of flower nodes, and number of seed pods in the sections supra. Alternatively, the data could be viewed as a one-way, completely randomized design. The latter approach could be valid if one assumes that there are no significant differences among blocks (replicates) and that differences between treatments are constant from one block to another (i.e., the block-by-treatment interaction is not significant). This was not the case, however, with the results presented in earlier sections. The analysis of variance results for each of the four dependent variables showed statistically significant (P less than 0.05) block-to-block variation, although this effect was much weaker (P = 0.085) for flower rating than for the other response variables.

By "pooling" the effects due to blocks and block-by-treatment interaction with the residual sum of squares and using the latter as the "appropriate" term to test for treatment effects, the power of detecting a treatment effect will be reduced. This is true if there is significant block-to-block variation and/or if there is a significant block-by-treatment interaction.

One may consider that blocks (replicates) 5 and 6 were different from blocks 1 and 4, because blocks 5 and 6 were planted later. Plants in these blocks, therefore, were in an earlier stage of development at the end of the experiment than plants in blocks 1-4, and were sprayed when they were one week older than plants in blocks 1-4. A re-analysis of the flower rating data, excluding blocks 5 and 6, was therefore performed (Table XLIII).

TABLE XLIII.

ANALYSIS OF VARIANCE: FLOWER RATING (1-4 REPLICATES).

RANDOMIZED BLOCK ANALYSIS OF SOYBEAN DATA

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: FRATING

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR F	R-SQUARE	C.V.
MODEL	31	85.00052705	2.74195249	1.79	0.0084	0.181560	23.8036
ERROR	250	383.16810967	1.53267244		ROOT MSE		FRATING MEAN
CORRECTED TOTAL	281	468.16863672			1.23801149		5.20094563

SOURCE	DF	TYPE I SS	F VALUE	PR F	DF	TYPE III SS	F VALUE	PR F
REP	3	3.34081032	0.73	0.5370	3	4.28010506	0.93	0.4264
TRT	7	49.73290209	4.64	0.0001	7	45.29704776	4.22	0.0002
REP*TRT	21	31.92681464	0.99	0.4738	21	31.92681464	0.99	0.4738

TESTS OF HYPOTHESES USING THE TYPE III MS FOR REP*TRT AS AN ERROR TERM

SOURCE	DF	TYPE III SS	F VALUE	PR F
REP	3	4.28010506	0.94	0.4397
TRT	7	45.29704776	4.26	0.0045

The analysis of variance results again indicat a strong treatment effect (P = 0.003). That is, the probability of erroneously concluding that there is a treatment effect is approximately 0.3 percent or about 3 in 1000. Furthermore, there does not appear to be any significant block-to-block variation (P = 0.47) or block-by-treatment interaction (P = 0.48).

It appears that blocks 5 and 6 were responsible for the significant block effect in the earlier analysis.

Table XLIV contains the Duncan's multiple range test results for the new analysis of flower rating which excludes blocks 5 and 6.

TABLE XLIV.

DUNCAN'S MULTIPLE RANGE TEST FOR VARIABLE: AVERAGE FLOWER RATING (1-4 REPLICATES).

ALPHA=.05 DF=250 MSE=1.53267

CELL SIZES ARE NOT EQUAL. HARMONIC MEAN OF CELL SIZES=34.8684

NUMBER OF MEANS	2	3	4	5	6	7	8
CRITICAL RANGE	0.589008	0.619378	0.63892	0.653601	0.665956	0.676054	0.684317

DUNCAN GROUPING*	MEAN	N	TREATMENT
A	5.7619	35	B1
A A	5.7083	32	B3
B A B A B A	5.5185	36	B5
B A B A B A C	5.3333	28	B7
B A C B A C B A C	5.1111	36	B2
B D C B D C B D C	5.0667	40	B4
D C D C	4.7838	37	B6
D D	4.4912	38	B8

*MEANS WITH THE SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT.

However, the original analysis (Table XXXII) should be considered as more appropriate than Table XLV for drawing conclusions. Even though there is block-to-block variation when blocks 5 and 6 are included, this effect is accounted for in the earlier analysis and allows the researcher to use all of the data to estimate the treatment effect. In fact, the treatment effect is stronger when blocks 5 and 6 are included in the analysis.

6.12. DEMONSTRATION OF THE INHERITANCE OF AMS/VECTOR-INDUCED MALE STERILITY IN A SUBSEQUENT GENERATION OF CORN

The study described herein demonstrates the inheritance of AMS/vector-induced male sterility into a subsequent generation of corn. The experiment was conducted on a field site at a research station in Waimanalo, Hawaii. Four sets of corn were planted (Table XLV).

TABLE XLV.

CORNSEED SOURCES*

Set	Description
1	Seed from crosses of sterile plants from AMS/vector-treated Inbred line x untreated isogenic line.
2	Seed from self crosses of fertile plants from AMS/vector-treated Inbred line.
3	Seed from crosses between AMS/vector-treated Inbred line x non-isogenic untreated Inbred line.
4	Seed of Inbreds 1, 2, 4 from S_2, S_3, S_4 and S_5 generations.**

*See section 6.11.1, infra for a more detailed description.

**S_n refers to the nth seed generation.

The codes for inbred lines of corn which were used are described in Table XLVI.

TABLE XLVI.

INBRED CODES FOR LINES OF CORN

Code	Seed Company Variety Code
Inbred 1	A632Ht, Lot 950, Grade F
Inbred 2	B73Ht, Lot 4551ST, Grade 23-21F
Inbred 3	H95Ht, Lot 150, Grade MF
Inbred 4	Mo17Ht, Lot 055, Grade MF

Sets 1 and 4 were tested to specifically evaluate the heritability of AMS/vector-induced male sterility into a subsequent generation of corn.

The objective in testing Set 2 material was to determine if sterility is expressed in subsequent generations of selfed corn plants that originally failed to convert to steriles upon AMS/vector treatment.

In Set 3 testing, the goal was to determine if the AMS/vector-Inbred line x non-isogenic, untreated Inbred line derived F_1 seed expressed any sterility.

Results from the data in Set 1 indicated that AMS/vector-induced male sterility in corn was inherited into a subsequent generation of corn. Inbreds 2 and 4 showed more than 80% male sterility in this set.

In Set 2, sterility was expressed in a few plants of Inbred 1 (1.7% sterility) and Inbred 3 (3.4% sterility).

In Set 3, no male sterility was expressed.

In Set 4, Inbreds 1, 2 and 4 showed more than 90% male sterility.

The tassels that wer rated fertile, in general, had all the anthers fully dehisced. Two exceptions were fertil plants of Inbr ds 2 and 4 in Set 1, wh re the tassels had only 1-10 anthers emerging per spik let, which were dehiscing and shedding pollen. The rest of the anthers were enclosed in the spikelet and did not dehisce. The tassels of sterile plants showed no dehiscence of anthers, which for the most part were enclosed in the spikelet.

Microscopic observations revealed that anthers from tassels rated fertile had round, stainable pollen grains, while anthers from tassels rated sterile had irregularly-shaped, non-stainable, abnormal pollen. In tassels which were rated fertile and which had only a few anthers dehisced, abnormal pollen was abundant in the non-dehisced anthers, while normal pollen was abundant in dehisced anthers.

6.12.1. MATERIALS AND METHODS

6.12.1.1. CORN SEED SOURCES

Prior to silking, the ears of corn plants of all the four genotypes in the experiment described in Section 6.9, supra, were covered with shoot tip bags. Three types of crosses were performed after the ears had silked. the seed derived from the three different crossing patterns constituted the seed for Sets 1-3 of the current experiment. The nature of these three sets and an additional set were as follows:

Set 1: After any male steriles were identified for each inbred strain, shoot tip bags were removed and the silks of the ears on the sterile plants were dusted with pollen derived from untreated isogenic Inbred

genotypes planted separately in the field. Ears were harvest d at maturity. Seed was separated from cobs and dried to 15% moisture. This seed (referred to as synthetic 1 or S_1) was designated as Set 1.

Set 2: AMS/vector-treated plants that produced pollen (fertiles) were selfed. The seed derived from such self-crosses was designated as Set 2.

Set 3: Crosses were made between AMS/vector-treated male-sterile Inbred lines and non-isogenic, untreated Inbred lines. The crosses were as follows: treated Inbred 1 X untreated Inbred 3; treated Inbred 2 X untreated Inbred 4; and treated Inbred 4 X untreated Inbred 2. The seed derived from such crosses was designated Set 3.

Set 4: Seed was generated, comprising four generations of each of three AMS/vector-treated, male-sterile Inbreds (1, 2 and 4) that were crossed to untreated isogenic lines. This seed, comprising S_2 - S_5 generations, was designated Set 4.

For sets 1-3, seed from each ear was shelled and packed in a seed packet. Each seed packet was given a treatment designation corresponding to the origin of the seed. For example, for Sets 1 and 2, a treatment designation of I_1 R_1 B_1 meant Inbred 1, treated with AMS/vector treatment B_1 from replicate 1 of the experiment described in Section 6.9. An example of treatment designation for Set 3 is T I_1 B_1 X UT I_3 corresponding to

a cross betw n AMS/vector-treat d (B_1 treatment) Inbr d 1 and untreated Inbred 3. Examples of Set 4 treatment designations include $I_1 S_2$, or I_2S_4 corresponding to the S_2 generation of Inbr d 1 or the S_4 gen ration of Inbred 2, respectively.

Sets 1 to 3 were prepared for ear to row planting with two replicates. Set 4 was also ear to row, but was planted only as one replicate.

6.12.1.2. PREPARATION OF SEED FOR PLANTING

For Sets 1-3, the seed derived from each ear was counted in two lots of 32 seeds each, and each lot was planted in a replicate. In instances where a single ear did not produce more than 32 seeds, only one replicate was planted.

The seed was coated with Captan, a wettable fungicide (Dragon Chemical Corporation, Roanoke, Virginia). Captan was mixed with water to make a thin paste. Four full tablespoons of Captan were mixed in 1 liter of water, and the solution was kept agitated with a magnetic stirrer. Thirty-two seeds from each packet were emptied into a tea strainer, which was dipped in the Captan-water mix. Excess Captan was strained off, the seed was placed on a paper towel to remove the excess moisture on the surface and was allowed to dry for 2 hours. The Captan-coated seed was then packed in paper bags, previously labeled with the appropriate treatment number. The seed was carefully packed and hand carried to Hawaii.

6.12.1.3. CHARACTERISTICS OF FIELD SITE

The field site was at Waimanalo research station, which is within the Waimanalo Village boundary in Hawaii. The research station is located at an elevation of 20 meters above the sea level, on a plane three miles

from the Pacific Ocean at 21 N latitude. The soil was Vertic Haplustoll derived from coral and lava intrusions and is considered prime farmland. The pH of the soil averages 6.0. Mean annual temperature at the station is 24°C, with monthly averages ranging from 22 to 27°C. Average annual rainfall is 1320 mm but monthly averages range from 10 to 180 mm. Rains are more frequent in winter months. Day lengths range from 10.8 to 13.2 hours. Winds are for the most part continuing and gentle at 8-15 km/hr but sometimes reach 25-30 km/hr. Incident light values average over 540 cal/cm^2/day, but can be as low as 220 cal/cm^2/day in cloudy winter months.

6.12.1.4. FIELD PREPARATION AND MANAGEMENT

A field site 105 feet x 120 feet was plowed, disked and rototilled. A basal fertilizer application consisting of NPK (nitrogen-phosphorus-potassium) in the ratio of 150:90:60 kg/ha was made using a fertilizer applicator. Another dose of 80 kgN/ha was applied between rows four weeks after emergence. The field was irrigated on an as needed basis on any Monday, Wednesday, or Friday.

6.12.1.5. EXPERIMENTAL DESIGN

The design was a randomized complete block design with ear to row planting done in two replicates for Sets 1, 2 and 3. Set 4 was also ear to row, but planted only in one replicate. Within each block or replicate, treatments (ear to row) were completely randomized. Each treatment was planted as a 10 foot row in each replicate. Thirty-two seeds were planted per row, with approximately 4 inch spacing between each seed. Planting within each block was done in tiers, 20 rows per each tier. Within each tier, the rows were separated by 3 foot spacing. The spacing between each tier was alternated as 2 feet, 4 feet, 2 feet, 4 feet, and so on. Where two tiers were

separated by 4 spacing, Pioneer hybrid 304C was planted as a cross row. No planting was done when the space between two tiers was 2.

6.12.1.6. FIELD PLANTING

One seed packet was assigned for each row according to the designated random number. Planting was done by hand using a push planter. Six persons, each with one planter, participated in planting at a given time. Each individual planted one row at a given time. After each row, the planters were cleaned by hand to ensure removal of dirt, etc. before moving on to plant another row. Planting was done on tier after tier, for example, rows 1-20 in the tier 1 were planted first before moving on to rows 21-40 in the tier 2. All rows in replicate 1 of Set 1 were planted before replicate 2 of Set 1. Set 2 planting commenced only after completion of Set 1 planting. Similarly, Set 3 was planted after completion of Set 2. Set 4 was planted in one tier of 12 rows, treatments being randomized within these 12 rows. After finishing planting of Sets 1-4, the untreated Inbreds 1-4 were planted in rows (one Inbred in each row), each row 100 feet long, to serve as pollen source for crossing. Another four rows of untreated Inbreds 1-4 were planted a week later as pollen source. On three sides of the experimental plots, a six row border was planted with Pioneer 304C. The fourth side was planted to corn four weeks later.

6.12.1.7. EXPERIMENTAL PARAMETERS

6.12.1.7.1. VISUAL RATING OF POLLEN

Stand counts were made a week after the emergence of corn plants. Plants that were severely dwarfed and/or heavily infested with virus or diseases,

and the dead plants were discarded prior to rating. The remaining plants were counted and rated for pollen fertility or sterility.

After the tassels had emerged out of the flag leaf (and after the ears had begun to silk), a black cardboard paper was placed under the tassel and the latter was shaken. If the tassels were shedding pollen on the black paper, the tassel was rated as fertile. Those tassels that did not show visible pollen on the black paper were rated as sterile. The fertile tassels were tagged with a red twine and the sterile tassels with a yellow twine. Those tassels that were deemed doubtful as to their pollen shedding were not rated, but were tagged with both yellow and red twines. The pollen rating was done from 8 AM to 12:30 PM every day, and was continued for three weeks. All the 4 sets were surveyed and rated every day, and all the tassels were checked to reconfirm their previous days' ratings. Those tassels with both yellow and red tags were rated as fertile or sterile, when the rating criteria were clearly met. Field observations on sterile tassels of Inbreds 1 and 2 with the yellow-colored tags are illustrated in Figures 14 and 15, respectively. At the end of the rating period, plants with yellow tags (steriles) and plants with red tags (fertiles) and total number of plants were counted in each row..

6.12.1.7.2. MICROSCOPIC OBSERVATIONS ON ANTHER AND POLLEN CHARACTERISTICS

Representative examples of fertile and sterile tassels for each Inbred were collected from the field. Anthers were dissected from the tassel and stained with acetocarmine. Morphological features of anthers and pollen were noted for each representative example.

6.12.1.7.3. RATING FOR PLANT HEIGHT, EAR HEIGHT, AND DAYS TO 75% SILKING

Plant height, ear height, and days to 75% silking were r cord d for each row. Plant height was measured on one average plant within a row. Plant height was measured in inches as the height from the ground level to the top of the tassel. Ear height was measured in inches from the ground level to the ear base. Ear height was measured on one average plant within a row. When 75% of the plants within a row showed silks on the ears, the date and month of that particular day was noted. Number of days from planting to 75% silking within a row constituted the number for days to 75% silking.

6.12.1.8. PROCEDURE FOR CROSSING

All the ears of plants within Sets 1-4 were covered with white transparent shoot tip bags as soon as the ears were visible and before the ears showed silks. Silks on each ear were cut with a scissor a day before crossing. At the time of crossing, the top of the shoot tip bag was torn off and the pollen dusted on the silks. The ears (with the remaining shoot tip bags still attached to the ear) were covered with the pollination bags. The bag was labeled with the Inbred number, the pollen source, the date the cross was made, and the name of the person who performed the cross. The nature of the crosses made in Sets 1-4 are summarized in Table XLVII.

TABLE XLVII.

CROSSES MADE IN CORN SETS 1-4

Set	Crosses
1	i) Sterile plants of Inbred line x non AMS/vector isogenic line.
	ii) Self crosses of a few fertile plants.
2	i) Sterile plants of Inbred line x non AMS/vector isogenic line.
	ii) Self crosses of a few fertile plants.
3	i) A few self crosses.
4	i) Sterile plants of Inbred line x non AMS/vector isogenic line.
	ii) Self crosses of a few fertile plants.

6.12.1.9. DATA COLLECTION AND STATISTICAL ANALYSIS

For each treatment row, data on total plant count, total plants rated, number of fertile plants, number of sterile plants, percentage of sterile plants, plant height, ear height, and days to 75% silking were recorded.

Statistical analyses were performed according to a completely randomized block design for Set 1. For Sets 2-4, means and standard deviations were provided for each treatment.

6.12.2. RESULTS AND DISCUSSION

6.12.2.1. INHERITANCE OF MALE STERILITY

Evaluation of the inh ritance of AMS/vector-induced male sterility into a subsequent generation of corn was examined in Sets 1 and 4.

6.12.2.1.1. SET 1

Seed was derived from male sterile plants (induced by AMS/vector) crossed with pollen from an untreated isogenic Inbred genotype. Inheritance of AMS/vector-induced male sterility was evident in Inbreds 2 and 4 with more than 80% of the plants being sterile (Tables XLVIII, XLIX). In Inbred 1, only 17% of the total plants were male sterile.

TABLE XLVIII.

SIGNIFICANCE LEVELS FOR BLOCKING FACTOR (REPLICATE), MAIN EFFECTS (INBRED AND TREATMENT) AND INTERACTION FROM ANALYSIS OF VARIANCE USING RANDOMIZED BLOCK DESIGN, BY DEPENDENT VARIABLE[a]

	Percent Sterility	Plant Height	Ear Height	Days To 75% Silking
Replicate (Block) (1, 2)	(.1245)	(.0002)**	(.0703)	(.0001)**
Inbred (1, 2, 4)	(.0001)**	(.0001)**	(.0001)**	(.0001)**
Treatment (B1, B4, B5, B6)	(.1817)	(.2919)	(.1175)	(.1786)
Inbred x Treatment	(.1038)	(.2090)	(.5284)	(.0158)*

[a]Highly significant results (P less than or equal to .01) indicated by "**"; P less than or equal to 0 indicated by "*" (Set 1)

TABLE XLIX.

RESULTS OF DUNCAN'S MULTIPLE RANGE TEST ON CLASSIFICATION VARIABLE INBRED FOR EACH OF THE DEPENDENT VARIABLES[a]

Dependent Variable	Mean Values For Class Variable Inbred		
Percent Sterile	Inbred 2 (95.4)	Inbred 4 (85.3)	Inbred 1 (16.8)
Plant Height	Inbred 1 (69.4)	Inbred 2 (63.4)	Inbred 4 (61.9)
Ear Height	Inbred 2 (23.8)	Inbred 1 (21.5)	Inbred 4 (21.1)
Days to 75% Silking	Inbred 2 (74.2)	Inbred 4 (73.9)	Inbred 1 (69.4)

[a]Values underscored by the same bold line were not significantly different at P less than or equal to 0.05. (Set 1)

6.12.2.1.2. SET 4

Inheritance of male sterility was demonstrated for four generations (S_2, S_3, S_4, and S_5) in Inbreds 1, 2, and 4 of Set 4, with more than 90% of the plants from each generation being male sterile (Table L).

TABLE L.

PERCENT STERILITY, PLANT HEIGHT, EAR HEIGHT, AND DAYS TO SILKING FOR INBREDS 1, 2, AND 4 ACROSS FOUR GENERATIONS (SET 4)

INBRED	GENERATION	REPLICATE	STAND COUNT	PLANT COUNT	NUMBER OF FERTILES	NUMBER OF STERILES	PERCENT OF STERILES	PLANT HEIGHT	EAR HEIGHT	DAYS TO SILK
1	S2	1	16	13	0	13	100.0	50.0	12.8	75
1	S3	1	22	22	3	19	86.4	65.0	21.0	69
1	S4	1	20	17	2	15	88.2	66.2	20.0	69
1	S5	1	17	17	1	16	94.1	64.2	19.0	69
2	S2	1	16	16	1	15	93.8	73.0	25.0	75
2	S3	1	18	15	0	15	100.0	70.0	22.5	73
2	S4	1	21	18	0	18	100.0	66.5	23.0	75
2	S5	1	12	7	0	7	100.0	64.0	21.0	75
4	S2	1	13	12	1	11	91.7	59.3	19.0	
4	S3	1	9	9	0	9	100.0	69.5	20.0	
4	S4	1	11	10	2	8	80.0	66.0	17.9	
4	S5	1	8	7	0	7	100.0	54.7	19.5	

6.12.2.1.3. SET 2

In Set 2, sterility was xpressed in a few plants of Inbred 1 (1.7% st rility) and Inbred 3 (3.4% sterility) (Table LI). The s ed in this Set was derived from self crosses that originally failed to convert to steriles upon AMS/vector treatment.

TABLE LI.

MEANS, STANDARD DEVIATIONS, AND
SAMPLE SIZE (N) FOR DATA IN SET 2
FOR RESPONSE VARIABLES BY INBRED GROUP

Inbred	Dependent Variable			
	Percent Sterility	Plant Height	Ear Height	Days To 75% Silking
1	1.7 ± 6.5 n = 31	66.9 ± 4.6 n = 31	20.3 ± 2.7 n = 31	71.4 ± 1.9 n = 31
3	3.4 ± 6.8 n = 17	58.9 ± 3.7 n = 17	15.6 ± 2.5 n = 17	71.9 ± 1.0 n = 17
4	0.0 ± 0.0 n = 2	68.2 ± 6.3 n = 2	19.3 ± 0.8 n = 2	72.0 ± 0.0 n = 2

6.12.2.1.4. SET 3

No male sterility was expressed in th hybrids derived from cross s b tween AMS/vector-tr ated Inbred genotypes and non-isogenic, untreated Inbred lin s (Table LII).

TABLE LII.

MEANS, STANDARD DEVIATIONS, AND
SAMPLE SIZE (N) FOR DATA IN SET 3
FOR RESPONSE VARIABLES BY INBRED CROSSES

	Dependent Variable			
Inbred	Percent Sterility	Plant Height	Ear Height	Days To 75% Silking
1 X 3	0.0 ± 0.0 n = 12	94.1 ± 5.8 n = 12	28.8 ± 3.2 n = 12	68.4 ± 0.5 n = 12
2 X 4	0.0 ± 0.0 n = 26	91.2 ± 6.1 n = 26	32.5 ± 3.7 n = 26	69.3 ± 1.1 n = 26
4 X 2	0.0 ± 0.0 n = 6	92.9 ± 2.9 n = 6	31.0 ± 3.8 n = 6	69.3 ± 1.4 n = 6

6.12.2.1.5. UNTREATED CONTROLS

No mal sterility was observed in the non-AMS/vector (untreated control) plants of Inbreds (Table LIII).

TABLE LIII.

PERCENT STERILITY, PLANT HEIGHT, EAR HEIGHT, AND DAYS TO 75% SILKING IN THE NON-AMS/VECTOR (UNTREATED CONTROLS) MATERIAL OF INBREDS 1, 2, 3, AND 4

INBRED	TOTAL PLANTS	FERTILES	STERILES	%STERILE	PLANT HEIGHT	EAR HEIGHT	DAYS TO SILKING
1	241	241	0	0	69	20	71
2	225	225	0	0	61	20	74
3	310	310	0	0	55	17	74
4	204	204	0	0	64	24	74

6.12.2.2. MORPHOLOGICAL FEATURES OF POLLEN FERTILITY AND STERILITY

6.12.2.2.1. VISUAL FEATURES

Visual observations of tassel morphology were made in the representative fertile and sterile plants of each inbred (Figs. 16, 17).

6.12.2.2.1.1. SET 1

In Set 1, Inbreds 2 and 4 formed three different types of tassels. In the first type, no anthers emerged out of the spikelet and the tassels showed no pollen shedding when they were shaken. Such tassels were rated as sterile. In the second type, only 1-10 anthers emerged out of a tassel, dehisced, and shed pollen. These were rated as fertile. In the third type, all the anthers in a tassel emerged out of the spikelets, dehisced, and shed profuse pollen. These were also rated as fertile. There were only two tassels of each of Inbred 2 and 4 in Set 1, which fell into the latter category.

In Inbred 1, plants rated sterile had no visible anthers on the tassel and there was no pollen shed. The plants rated fertile had anthers which had emerged out of the spikelet, and exhibited profuse pollen shedding from the anthers. Pollen shedding was delayed in some plants of Inbred 1, which necessitated revision of rating in some instances.

6.12.2.2.1.2. SET 2

Plants belonging to all the Inbreds in this set which were rated fertile had tassels with anthers dehisced, and shed pollen profusely. Those that were rated sterile had all the anthers enclosed within the spikelet, and exhibited no pollen shedding.

6.12.2.2.1.3. SET 3

All th plants in Set 3 had tassels with dehisced anthers that shed pollen profusely.

6.12.2.2.1.4. SET 4

Tassels which were rated sterile had no anthers emerging out of the spikelet or shedding pollen. Those rated fertile had fully dehisced anthers and profuse pollen shed.

6.12.2.2.1.5. UNTREATED CONTROLS

All the tassels had anthers fully dehisced, with profuse pollen shedding.

6.12.2.2.2. MICROSCOPIC FEATURES

Anthers from representative examples of tassels rated fertile or sterile were stained with acetocarmine, and the preparations were examined for differences in the characteristics of anthers and pollen (Figs. 18-22).

6.12.2.2.2.1. SET 1

Tassels where the anthers were fully dehisced and pollen washed profusely, showed typically large, round, normal-looking pollen with cytoplasm densely stained with acetocarmine.

Tassels where only 1-10 anthers emerged out of the spikelet and dehisced, showed normal looking, round pollen grains with dense cytoplasm, only in the anthers that emerged and dehisced. In the same tassel, anthers which remained in the spikelet and failed to dehisce showed abnormal, irregularly shaped pollen, with very little stainable cytoplasm. A few of the undehisced anthers were bulged in the middle and the bulge was filled with normal-looking pollen grains, while the rest of the anther had abnormal pollen.

In Inbreds 1 and 2, tassels which were rated sterile had undehisced anthers containing abnormal pollen. Crushing the anthers with a glass rod facilitated the release of pollen grains from the anthers. However, in Inbred 4, no pollen grains were seen inside the anthers. Therefore, in all the three Inbreds, the block associated with male sterility appeared to be at the level of differentiation of sporogenous tissue. There was also a good correlation between lack of dehiscence of anthers and presence of abnormal pollen or absence of pollen.

6.12.2.2.2.2. SET 2

Microscopic observations revealed that all tassels rated fertile had anthers with large, round pollen grains with densely stained cytoplasm. The anthers from tassels rated sterile did not dehisce and had abnormal, irregularly shaped, non-stainable pollen.

6.12.2.2.2.3. SET 3

All tassels from Set 3 were rated fertile. The anthers dehisced, and pollen grains were round and had densely stained cytoplasm.

6.12.2.2.2.4. SET 4

Tassels rated fertile had anthers showing normal, round pollen with densely stained cytoplasm. Those tassels that were rated sterile had undehisced anthers and contained abnormal pollen.

6.12.2.2.2.5. UNTREATED CONTROLS

The pollen grains from the untreated control plants of all the four Inbreds were round with dense cytoplasm that stained deep red with acetocarmine.

6.12.2.3. STATISTICAL ANALYSIS OF THE DATA

6.12.2.3.1. SET 1

Highly significant differences were noticed between Inbreds for the percent sterility variable. Differences were also noted between Inbreds for plant height, ear height, and days to 75% silking characteristics. A strong to moderate effect between replicate plots (blocks) across all four dependent variables (P = 0.0001 to 0.1245) was observed, the least effect being in the percent sterility variable (Table XLVIII). No significant effect of the AMS/vector treatments (B1, B4, B5, and B6 of Section 6.9, supra) applied on plants in the previous generation, was apparent in this generation, for any of the four dependent variables. Duncan's multiple range test also conveyed similar trends (Table XLIV). Means of the percent sterile variable were significantly different for all the three inbreds. Plant height of Inbred 1 was significantly different from Inbreds 2 and 4. For the dependent variable days to silking, Inbreds 2 and Inbreds 4 were significantly different from Inbred 1.

6.12.2.3.2. SETS 2 AND 3

Means and standard deviations were calculated for all the dependent variables for Sets 2 and 3. In Set 2, Inbred 1 and 3 showed 1.7% and 3.4% male sterile plants, respectively. No male sterile plants were identified in Set 3. No noticeable trends for other dependent variables were apparent (Tables LI, LII).

6.12.2.3.3. SET 4

In the absence of any replication, the actual values were tabulated for this Set (Table L).

7. DEPOSITS OF SEEDS

The following seeds have been deposited with the American Type Culture Collection, Rockville, MD., and have been assigned the listed accession numbers:

Seed	Description	Accession Number
AMS 1.29	cross between alfalfa AMS/vector source 1.29 (derived from U.S.D.A. PI No. 223386) and a maintainer plant	40352
B73-AMS	male-sterile B73Ht variety of Zea mays L. corn; asexually induced to male sterility by treatment with AMS/vector	40350
Mo17-AMS	male-sterile Mo17Ht variety of Zea mays L. corn; asexually induced to male sterility by treatment with AMS/vector	40351
A632-AMS	male-sterile A632Ht variety of Zea mays L. corn; asexually induced to male sterility by treatment with AMS/vector	40349

The present invention is not to be limited in scope by the specific seeds deposited since the deposited embodiment is intended as a single illustration of one aspect of the invention and any seed which is functionally equivalent is within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. An AMS/vector comprising a cytoplasmic factor derived from a donor plant, which factor (a) is capable of asexually inducing heritable male sterility in a recipient plant; (b) is subsequently derivable from the recipient plant; and (c) is present in an extract of the donor plant or recipient plant, which extract further comprises a nucleic acid of about 1 X 10^6 dalton molecular weight and a particle of about 40-110 nanometers.

2. The AMS/vector of claim 1 in which the donor plant is an alfalfa plant.

3. The AMS/vector of claim 2 in which the alfalfa plant has U.S.D.A. Plant Introduction No. 172429.

4. The AMS/vector of claim 2 in which the alfalfa plant has U.S.D.A. Plant Introduction No. 173733.

5. The AMS/vector of claim 2 in which the alfalfa plant has U.S.D.A. Plant Introduction No. 221469.

6. The AMS/vector of claim 2 in which the alfalfa plant has U.S.D.A. Plant Introduction No. 223386.

7. The AMS/vector of claim 2 in which the alfalfa plant has U.S.D.A. Plant Introduction No. 243223.

8. The AMS/vector of claim 2 in which the alfalfa plant comprises plant AMS 1.29, as deposited with the ATCC and assigned accession number 40352.

9. The AMS/vector of claim 1 in which the donor plant is a corn plant.

10. The AMS/vector of claim 1 in which the donor plant is a soybean plant.

11. The AMS/vector of claim 1 in which the donor plant is a sorghum plant.

12. The AMS/vector of claim 1 in which the donor plant is a sunflower plant.

13. The AMS/vector of claim 1 in which the donor plant is a millet plant.

14. The AMS/vector of claim 1 in which the donor plant is a tomato plant.

15. A plant extract capable of inducing male sterility in a plant, comprising a non-lethal buffer and a cytoplasmic factor derived from a donor plant, which factor (a) is capable of asexually inducing heritable male sterility in a recipient plant; (b) is subsequently derivable from the recipient plant; and (c) is present in an extract of the donor plant or recipient plant, which extract further comprises a nucleic acid of about 1 X 10^6 dalton molecular weight and a particle of about 40-110 nanometers.

16. The extract of claim 15 in which the donor plant is an alfalfa plant.

17. The extract of claim 15 in which the donor plant is a corn plant.

18. Th extract of claim 15 in which the donor plant is a soyb an plant.

19. The extract of claim 15 in which the donor plant is a sorghum plant.

20. The extract of claim 15 in which the donor plant is a sunflower plant.

21. The extract of claim 15 in which the donor plant is a millet plant.

22. The extract of claim 15 in which the donor plant is a tomato plant.

23. A male-sterile plant comprising a plant that has been asexually induced to heritable male sterility by the AMS/vector of claim 1.

24. A male-sterile plant comprising a plant that has been asexually induced to heritable male sterility by the AMS/vector of claim 2.

25. A male-sterile plant comprising a plant that has been asexually induced to heritable male sterility by the AMS/vector of claim 3, 4, 5, 6, 7, or 8.

26. The plant of claim 23 which is an alfalfa plant.

27. The plant of claim 23 which is a corn plant.

28. The corn plant of claim 27 comprising B73-AMS, as deposited with the ATCC and assigned accession number 40350.

29. The corn plant of claim 27 comprising Mo17-AMS, as deposited with the ATCC and assigned accession number 40351.

30. The corn plant of claim 27 comprising A632-AMS, as deposited with the ATCC and assigned accession number 40349.

31. The plant of claim 23 which is a soybean plant.

32. The plant of claim 23 which is a sorghum plant.

33. The plant of claim 23 which is a sunflower plant.

34. The plant of claim 23 which is a millet plant.

35. The plant of claim 23 which is a tomato plant.

36. The plant of claim 23 which is a wheat plant.

37. The plant of claim 23 which is a cotton plant.

38. The plant of claim 23 which is a rice plant.

39. A progeny plant obtained by asexual propagation of the plant of claim 23, 24, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37 or 38.

40. The progeny plant of claim 39, in which the propagation is by cell culture methods.

41. The progeny plant of claim 39, in which the propagation is vegetative.

42. A seed resulting from a cross of the plant of claim 23 or 24 with a maintainer plant.

43. A seed resulting from a cross of the plant of claim 25 with a maintainer plant.

44. A seed resulting from a cross of the plant of claim 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37 or 38 with a maintainer plant.

45. A progeny plant produced by the seed of claim 42.

46. A progeny plant produced by the seed of claim 43.

47. A progeny plant produced by the seed of claim 44.

48. A method for asexually inducing male sterility in a recipient plant comprising applying the AMS/vector of claim 1 to such recipient plant.

49. A method for asexually inducing male sterility in a recipient plant comprising applying the AMS/vector of claim 2 to such recipient plant.

50. A method for asexually inducing male sterility in a recipient plant comprising applying the AMS/vector of claim 8 to such recipient plant.

51. A method for asexually inducing male sterility in a recipient plant comprising applying the extract of claim 15 to such recipient plant.

52. The method of claim 48, 49, 50 or 51 in which the application is by injection.

53. The method of claim 48, 49, 50 or 51 in which the application is by spraying.

54. The method of claim 48, 49, 50 or 51 in which the application is by use of tissue culture.

55. The method of claim 48, 49, 50 or 51 in which the application is by electroporation.

56. The method of claim 48, 49, 50 or 51 in which the recipient plant is an alfalfa plant.

57. The method of claim 48, 49, 50 or 51 in which the recipient plant is a corn plant.

58. The method of claim 48, 49, 50 or 51 in which the recipient plant is a soybean plant.

59. The method of claim 48, 49, 50 or 51 in which the recipient plant is a sorghum plant.

60. The method of claim 48, 49, 50 or 51 in which the recipient plant is a sunflower plant.

61. The method of claim 48, 49, 50 or 51 in which the recipient plant is a millet plant.

62. The method of claim 48, 49, 50 or 51 in which the recipient plant is a tomato plant.

63. The method of claim 48, 49, 50 or 51 in which the recipient plant is a wheat plant.

64. The method of claim 48, 49, 50 or 51 in which the recipient plant is a cotton plant.

65. The method of claim 48, 49, 50 and 51 in which the recipient plant is a rice plant.

66. A method for making an F_1 hybrid comprising crossing a paternal parent plant with the plant of claim 23 or 24 as maternal parent.

67. A method for making an F_1 hybrid comprising crossing a paternal parent plant with the plant of claim 25 as maternal parent.

68. A method for making an F_1 hybrid comprising crossing a paternal parent plant with the plant of claim 45 as maternal parent.

69. A method for making an F_1 hybrid comprising crossing a paternal parent plant with the plant of claim 46 as maternal parent.

70. A method for making an F_1 hybrid comprising crossing a paternal parent plant with the plant of claim 47 as maternal parent.

71. An F_1 hybrid made according to the method of claim 66.

72. An F_1 hybrid made according to the method of claim 67.

73. An F_1 hybrid made according to the method of claim 68.

74. An F_1 hybrid made according to the method of claim 69.

75. An F_1 hybrid made according to the method of claim 70.

76. The F_1 hybrid of claim 71 which is male sterile.

77. The F_1 hybrid of claim 72 which is male sterile.

78. The F_1 hybrid of claim 73 which is male sterile.

79. The F_1 hybrid of claim 74 which is male sterile.

80. The F_1 hybrid of claim 75 which is male sterile.

81. Th F_1 hybrid of claim 71 which is male fertile.

82. The F_1 hybrid of claim 72 which is male fertile.

83. The F_1 hybrid of claim 73 which is male fertile.

84. The F_1 hybrid of claim 74 which is male fertile.

85. The F_1 hybrid of claim 75 which is male fertile.

86. A seed of the F_1 hybrid of claim 71.

87. A seed of the F_1 hybrid of claim 72.

88. A seed of the F_1 hybrid of claim 73.

89. A seed of the F_1 hybrid of claim 74.

90. A seed of the F_1 hybrid of claim 75.

91. A seed of the F_1 hybrid of claim 76.

92. A seed of the F_1 hybrid of claim 77.

93. A seed of the F_1 hybrid of claim 78.

94. A seed of the F_1 hybrid of claim 79.

95. A seed of the F_1 hybrid of claim 80.

96. A seed of the F_1 hybrid of claim 81.

97. A seed of the F_1 hybrid of claim 82.

98. A seed of the F_1 hybrid of claim 83.

99. A seed of the F_1 hybrid of claim 84.

100. A seed of the F_1 hybrid of claim 85.

101. A method for inducing apomixis in a recipient plant comprising applying an effective amount of the AMS/vector of claim 1 to such recipient plant.

102. A method for inducing apomixis in a recipient plant comprising applying the AMS/vector of claim 2 to such recipient plant.

103. A method for inducing apomixis in a recipient plant comprising applying the AMS/vector of claim 8 to such recipient plant.

104. A method for inducing apomixis in a recipient plant comprising applying the AMS/vector of claim 15 to such recipient plant.

105. The method of claim 101, 102, 103 or 104, in which the application is by spraying.

106. The method of claim 101, 102, 103 or 104 in which the application is by injection.

107. The method of claim 101, 102, 103 or 104 in which the application is by use of tissue culture.

108. The method of claim 101, 102, 103 or 104 in which the application is by electroporation.

109. The method of claim 101, 102, 103 or 104 in which the recipient plant is an alfalfa plant.

110. The method of claim 101, 102, 103 or 104 in which the recipient plant is a corn plant.

111. The method of claim 101, 102, 103 or 104 in which the recipient plant is a sorghum plant.

112. The method of claim 101, 102, 103 or 104 in which the recipient plant is a sunflower plant.

113. The method of claim 101, 102, 103 or 104 in which the recipient plant is a millet plant.

114. The method of claim 101, 102, 103 or 104 in which the recipient plant is a tomato plant.

115. The method of claim 101, 102, 103 or 104 in which the recipient plant is a wheat plant.

116. The method of claim 101, 102, 103 or 104 in which the recipient plant is a cotton plant.

117. The method of claim 101, 102, 103 or 104 in which the recipient plant is a rice plant.

118. A method of making an apomitic hybrid which compris s treating a first parental plant line of said

hybrid with an effective amount of the AMS/vector of claim 1, and crossing said first parental lin with a second parental plant lin to obtain hybrid seed.

119. The method of claim 118 which includes the further step of growing the hybrid seed to produce mature plants, and identifying those plants having apomitic properties.

120. The method of claim 118 which comprises the further step of obtaining hybrid seed from the identified plants.

121. The method of claim 118, 119 or 120, wherein the plant is an alfalfa plant.

122. The method of claim 118, 119 or 120, wherein the plant is a corn plant.

123. The method of claim 118, 119 or 120, wherein the plant is a soybean plant.

124. The method of claim 118, 119 or 120, wherein the plant is a sorghum plant.

125. The method of claim 118, 119 or 120, wherein the plant is a sunflower plant.

126. The method of claim 118, 119 or 120, wherein the plant is a millet plant.

127. The method of claim 118, 119 or 120, wherein the plant is a tomato plant.

128. Th method of claim 118, 119 or 120, wherein the plant is a wheat plant.

129. The method of claim 118, 119 or 120, wherein the plant is a cotton plant.

130. The method of claim 118, 119 or 120, wherein the plant is a rice plant.

131. Hybrid seed produced by the method of claim 118.

132. Hybrid seed produced by the method of claim 120.

133. A hybrid plant produced by the method of claim 119, and direct descendants thereof.

134. The seed of claim 131 wherein the plant is selected from the group consisting of corn, alfalfa, soybean, sorghum, sunflower, millet, tomato, wheat, cotton and rice.

135. The seed of claim 132 wherein the plant is selected from the group consisting of corn, alfalfa, soybean sorghum, sunflower, millet, tomato, wheat, cotton and rice.

136. The plant of claim 133 wherein the plant is selected from the group consisting of corn, alfalfa, soybean, sorghum, sunflower, millet, tomato, wheat, cotton and rice.

137. A method of d livering a bioactive molecule intracellularly to a plant comprising applying the about

40-110 nanom ter particle associated with the AMS/vector of claim 1, which particle contains a bioactiv molecule.

138. A plant d livery system comprising an about 40-110 nanometer particle derivable from an alfalfa plant selected from the group consisting of plants having U.S.D.A. Plant Introduction Nos. 172429, 173733, 221469, 223386, and 243223.

139. A method of expressing a heterologous gene sequence in a plant comprising applying the about 1 X 10^6 dalton nucleic acid of claim 1, which nucleic acid comprises a heterologous gene sequence capable of being expressed in the plant.

140. A plant expression vector comprising an about 1 X 10^6 dalton molecular weight nucleic acid derivable from an alfalfa plant selected from the group consisting of plants having U.S.D.A. Plant Introduction Nos. 172429, 173733, 221469, 223386, and 243223.

141. A mutant, derivative, or fragment of the expression vector of claim 108.

FIG. 1A

FIG. 1B

FIG. 1C

FIG. 1D

FIG. 1E

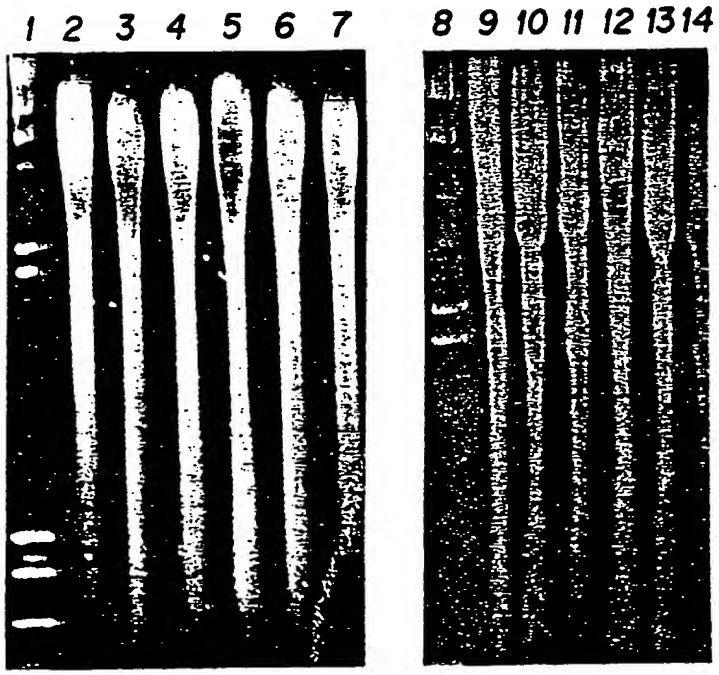

FIG. 2A

FIG. 2B

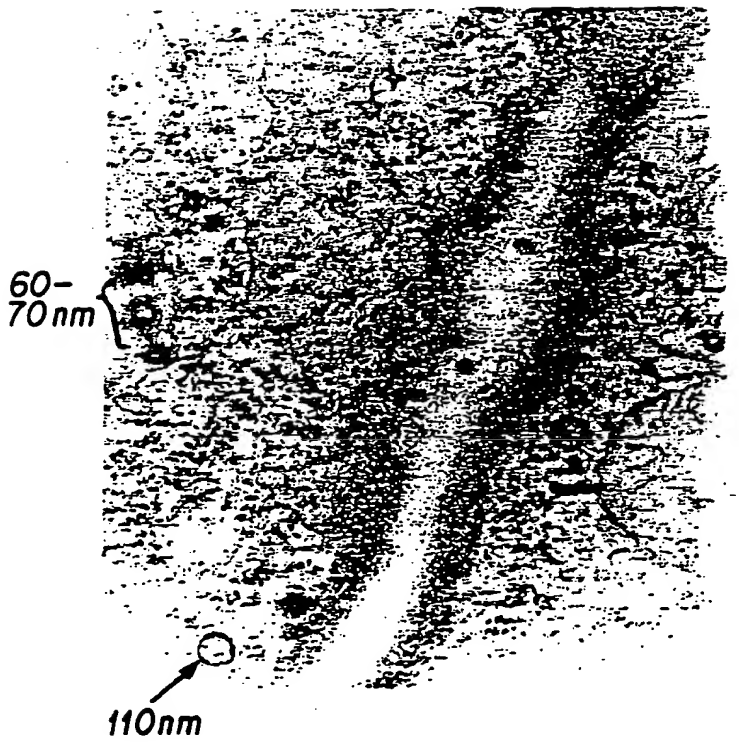

FIG. 2C

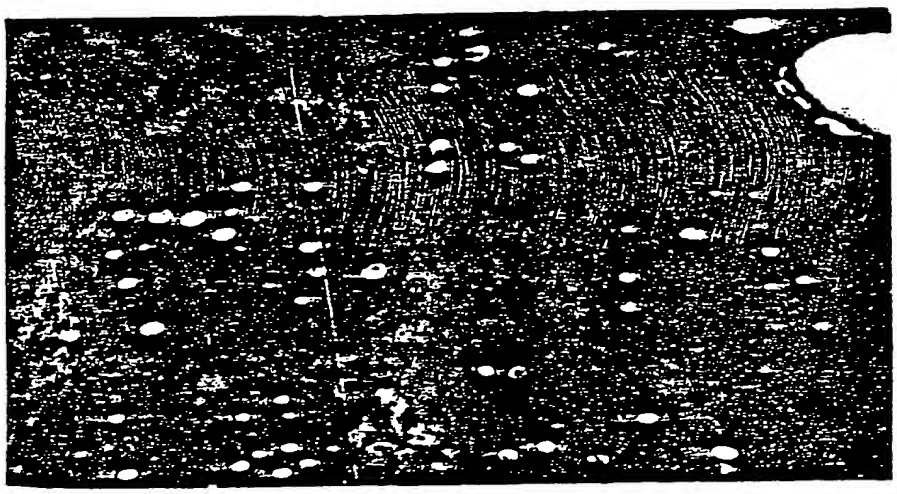

FIG. 3A

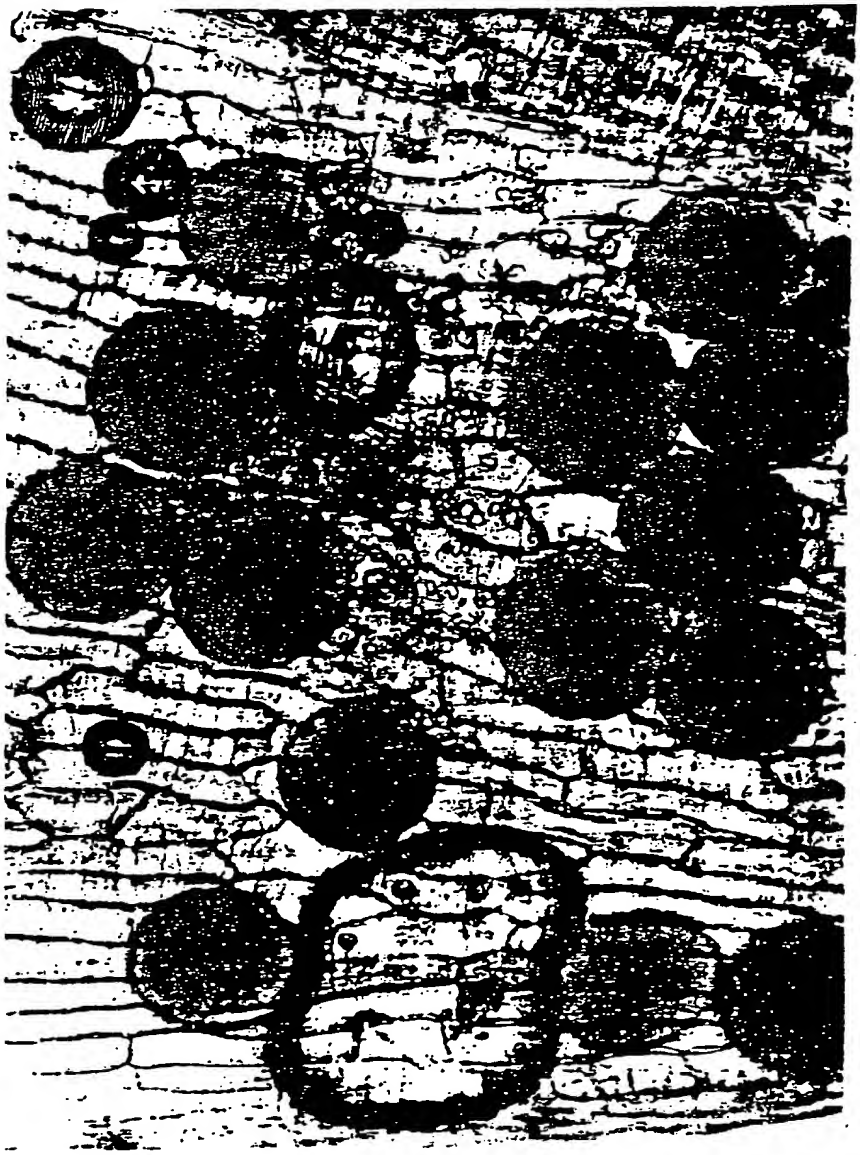

FIG. 3B

FIG. 3C

FIG. 3D

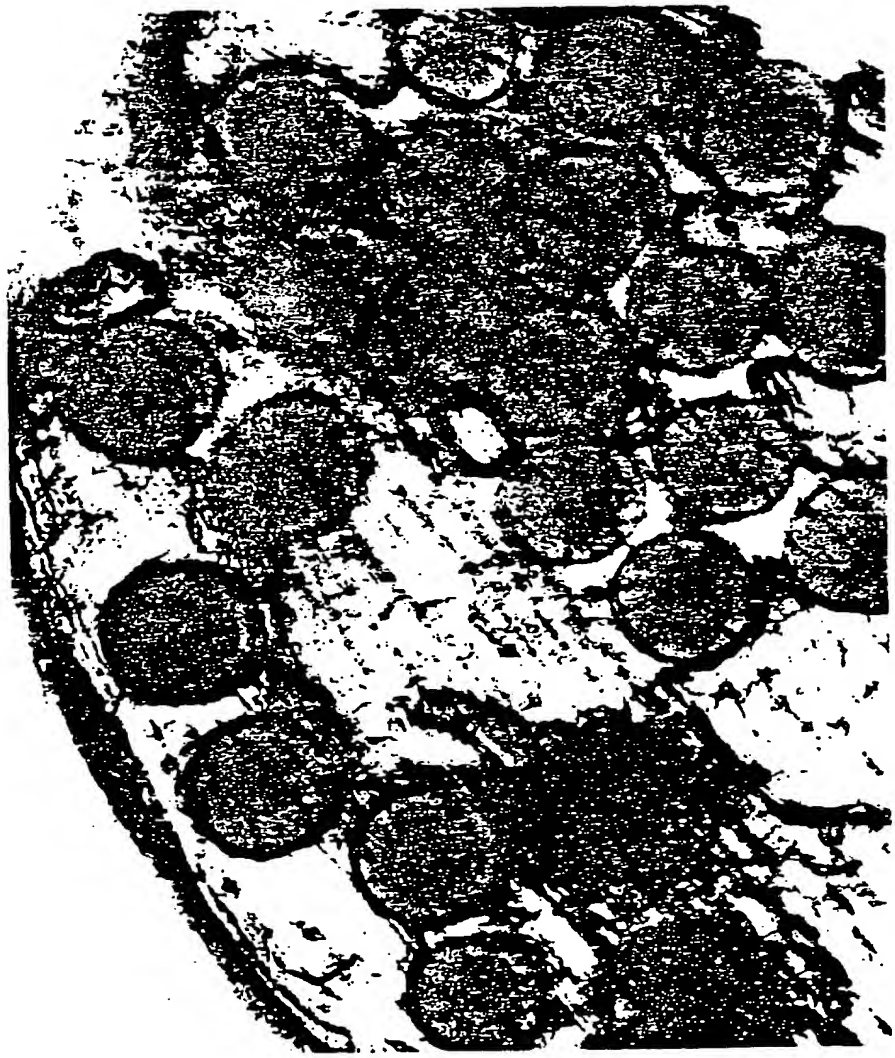

FIG. 4

FIG. 5A

FIG. 5B

FIG.6

FIG. 7

FIG. 8

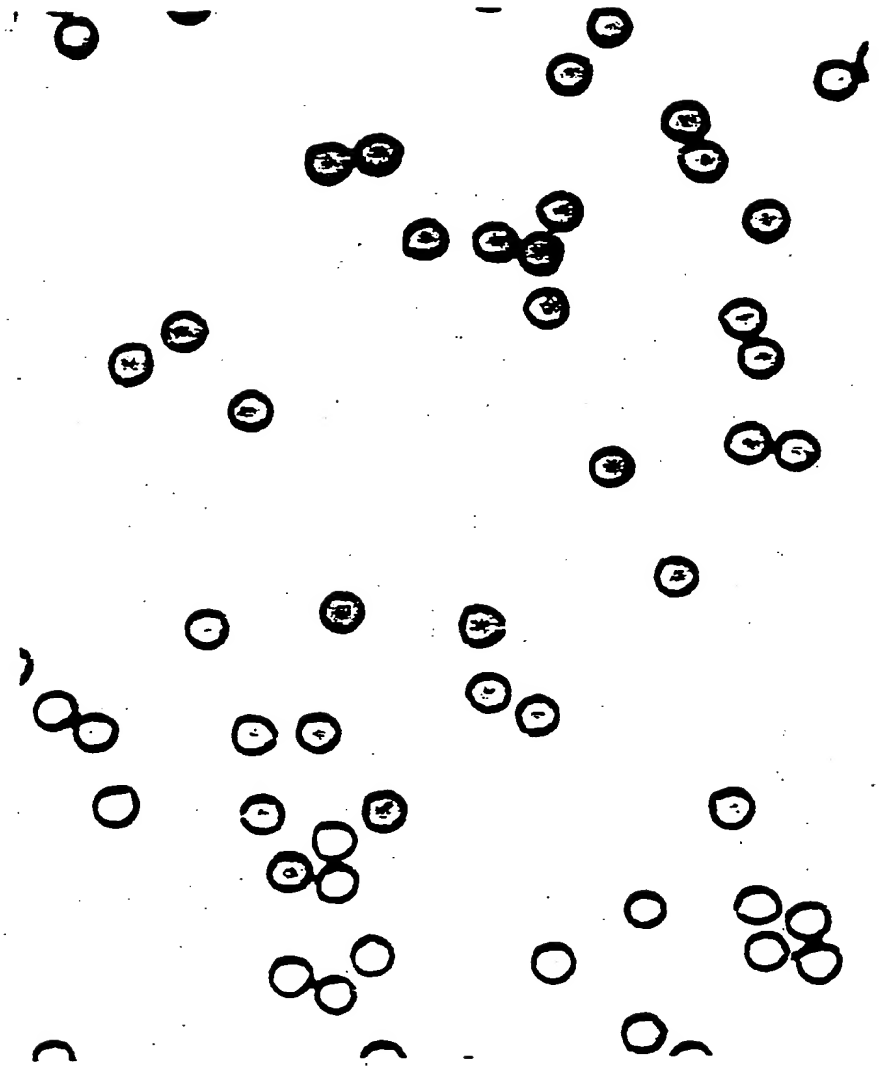

FIG. 9

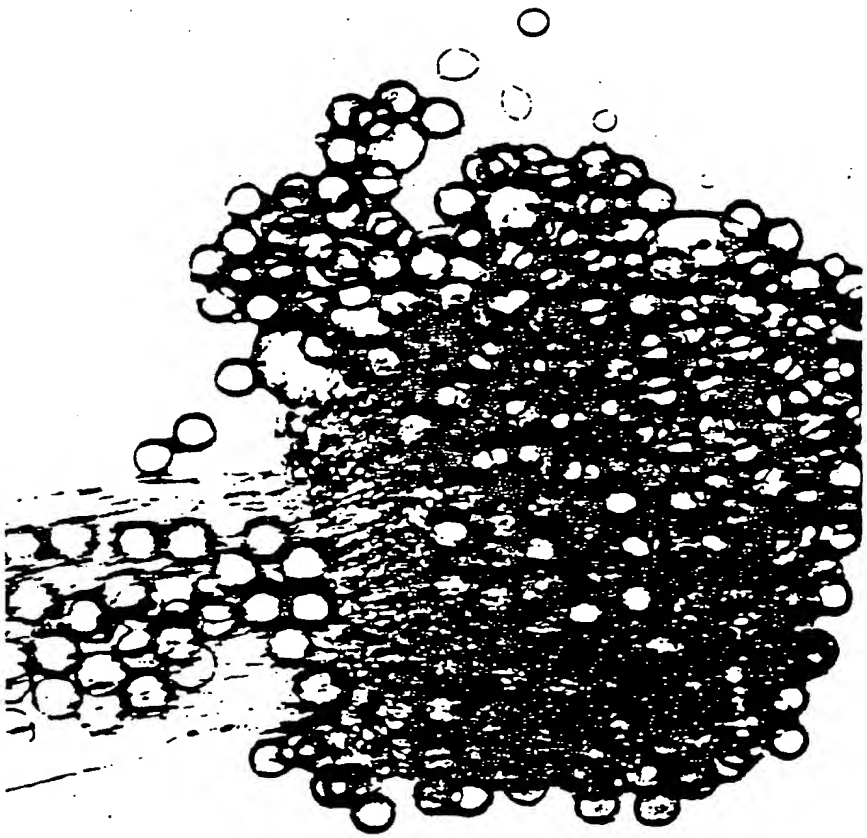

FIG. 10

FIG. 11

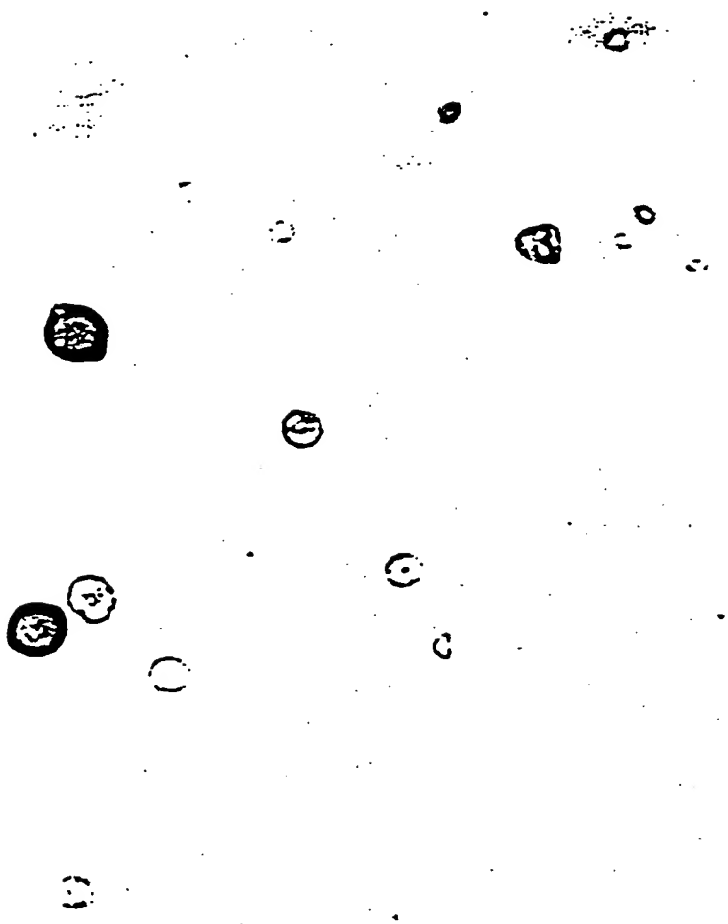

FIG. 12

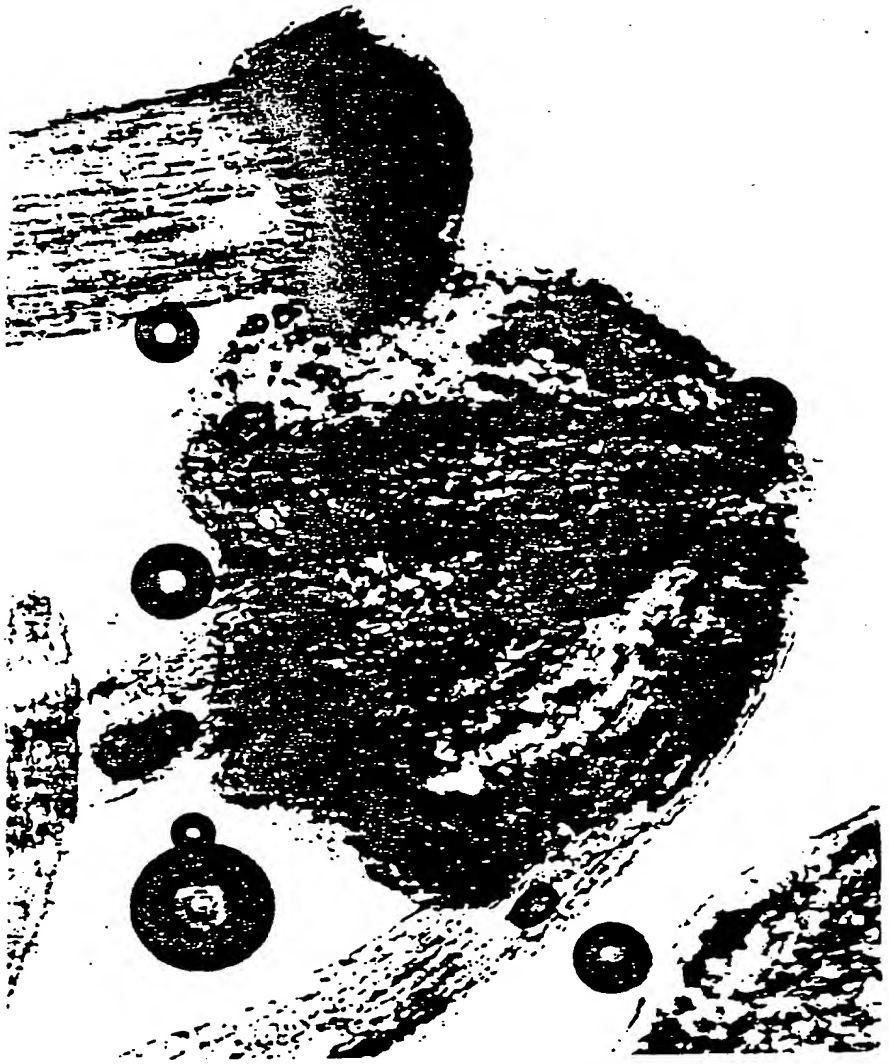

FIG.13

FIG. 14

FIG. 15

FIG. 16A

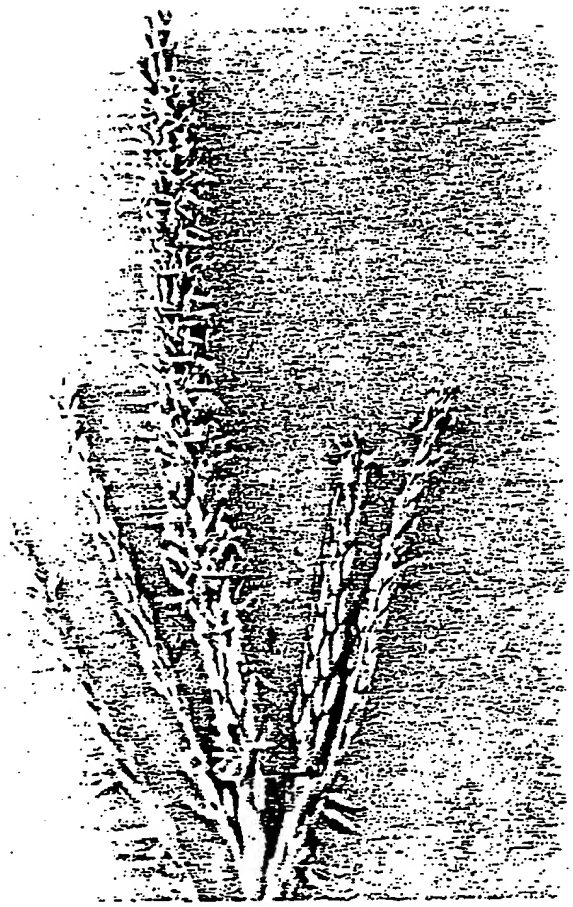

FIG. 16B

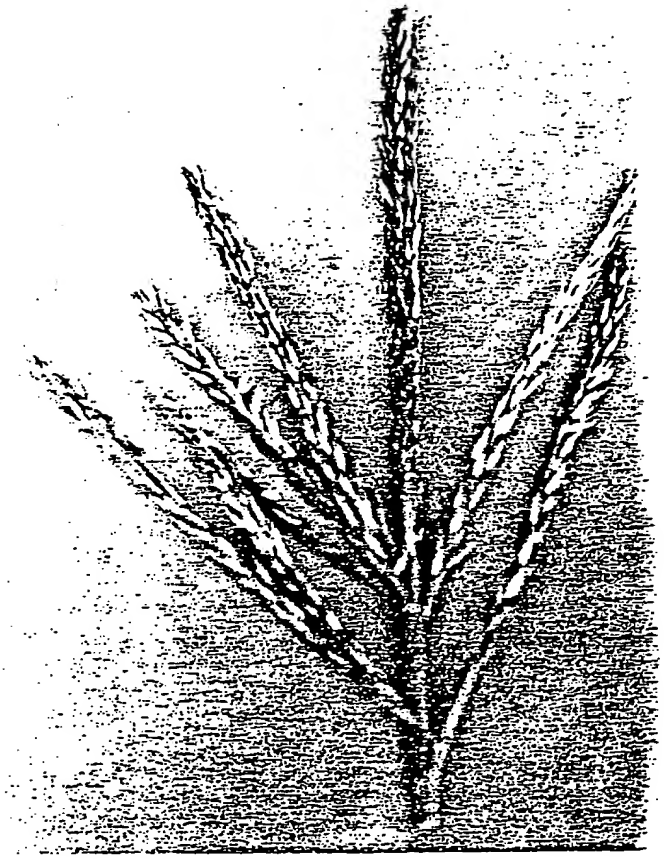

FIG. 16C

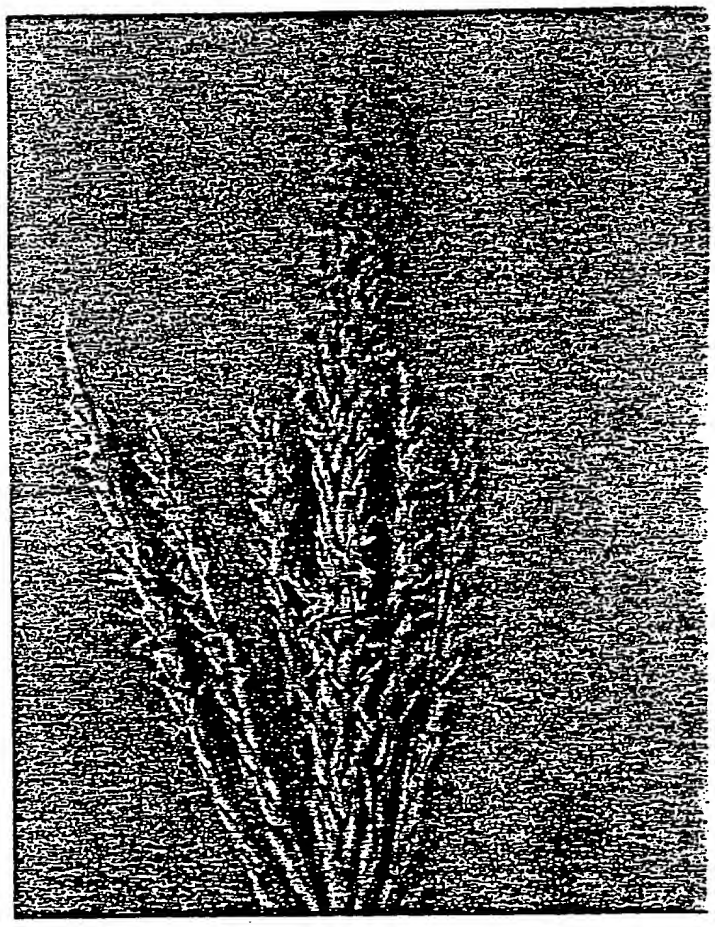

FIG. 16D

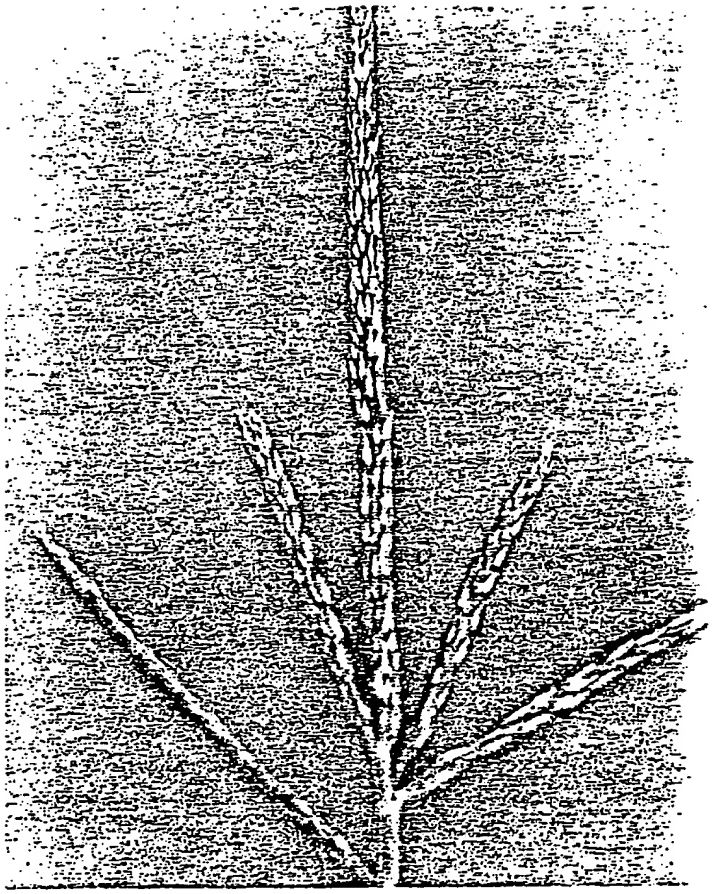

FIG. 17A

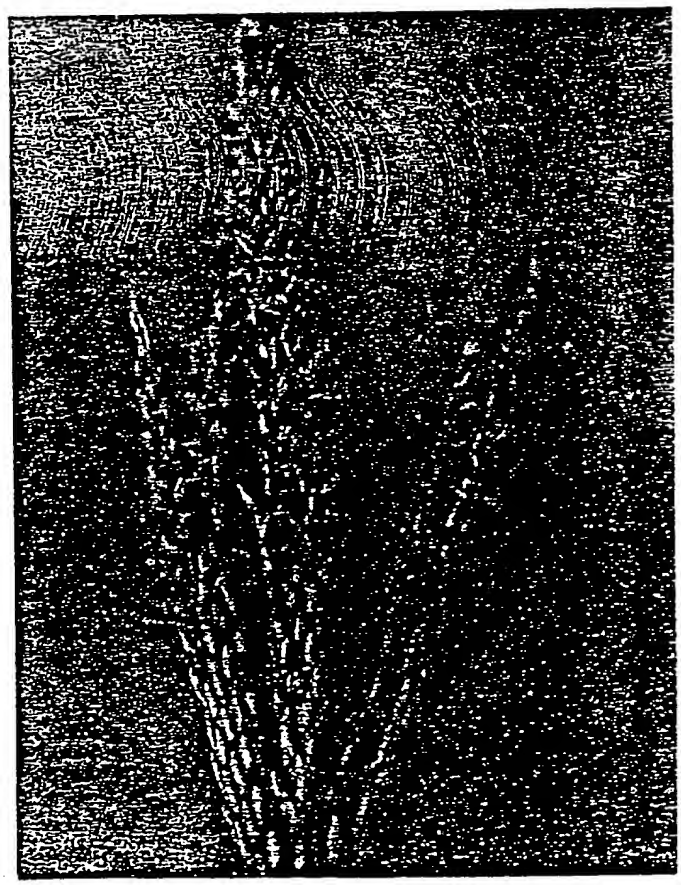

FIG. 17B

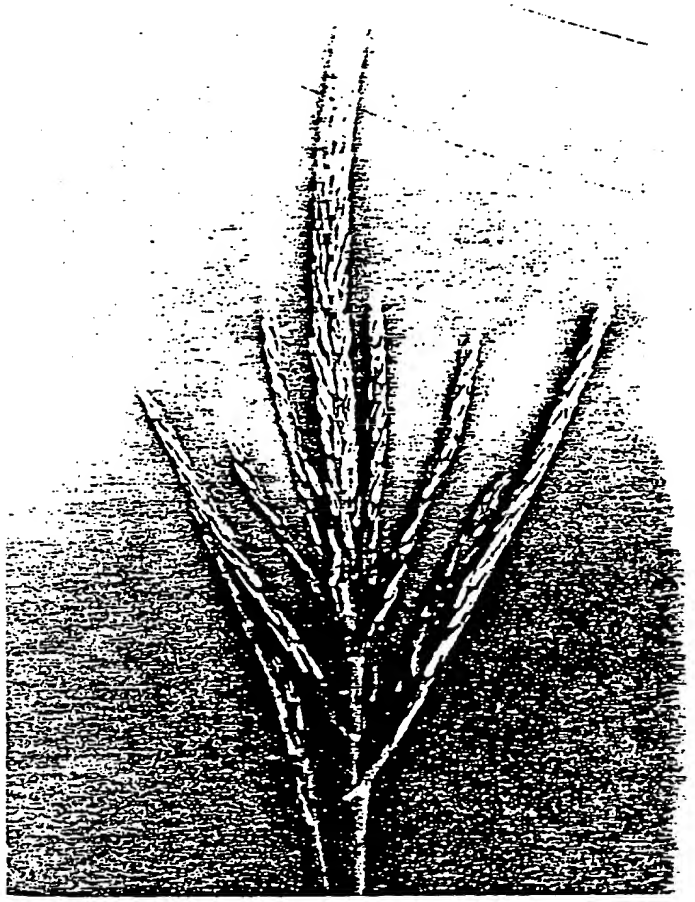

FIG. 17C

FIG. 17D

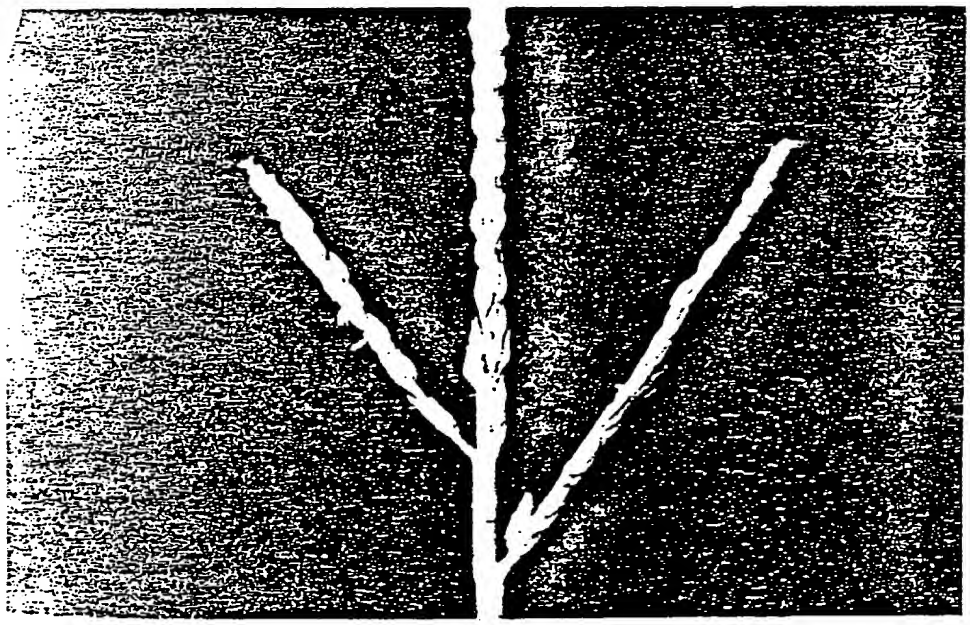

FIG.18A

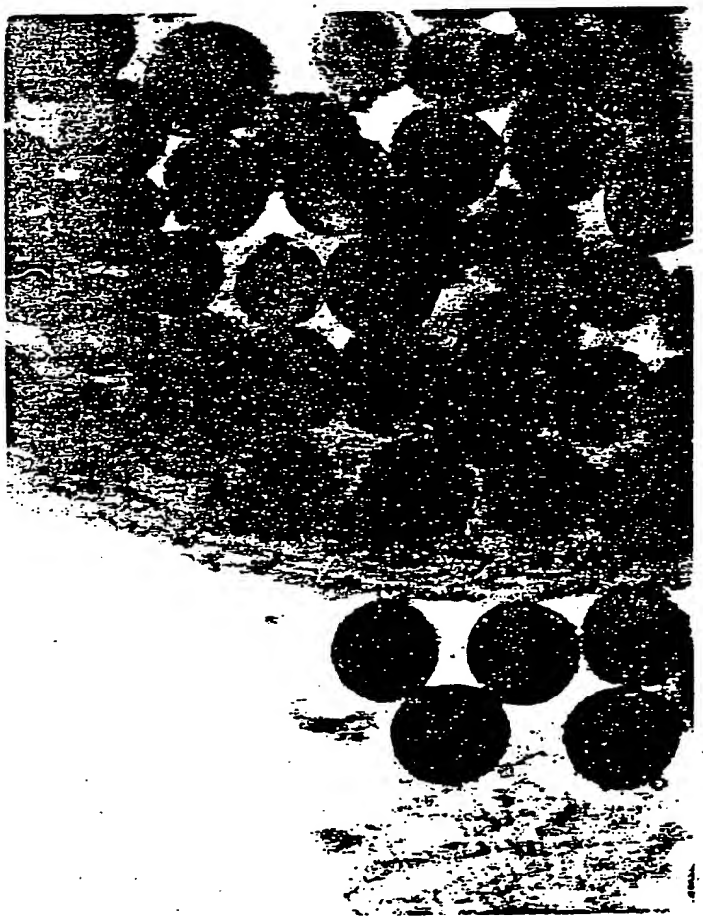

FIG. 18B

FIG. 18C

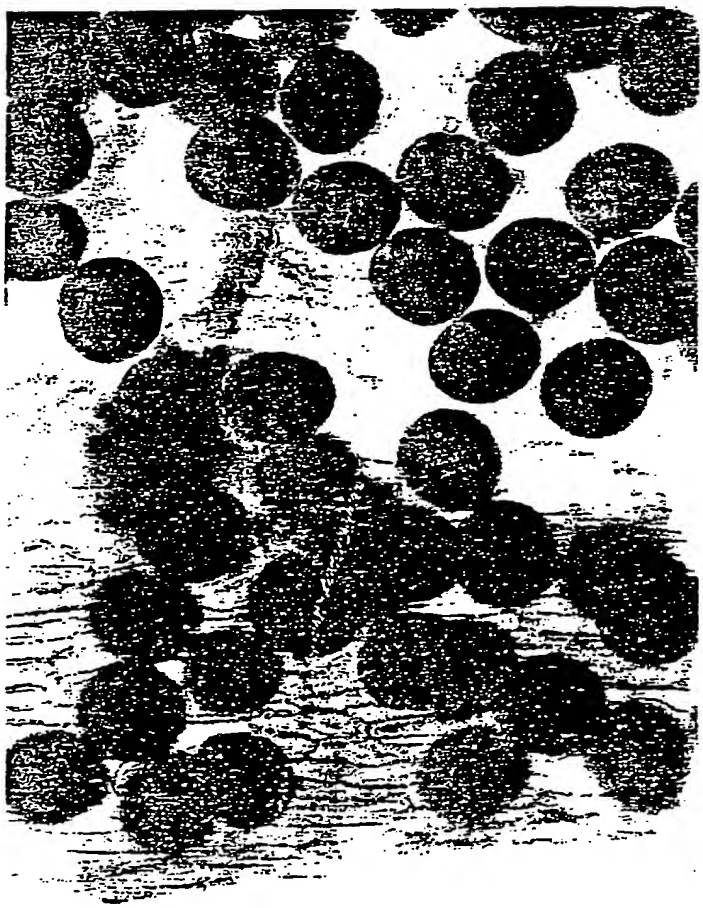

FIG. 18D

FIG. 19A

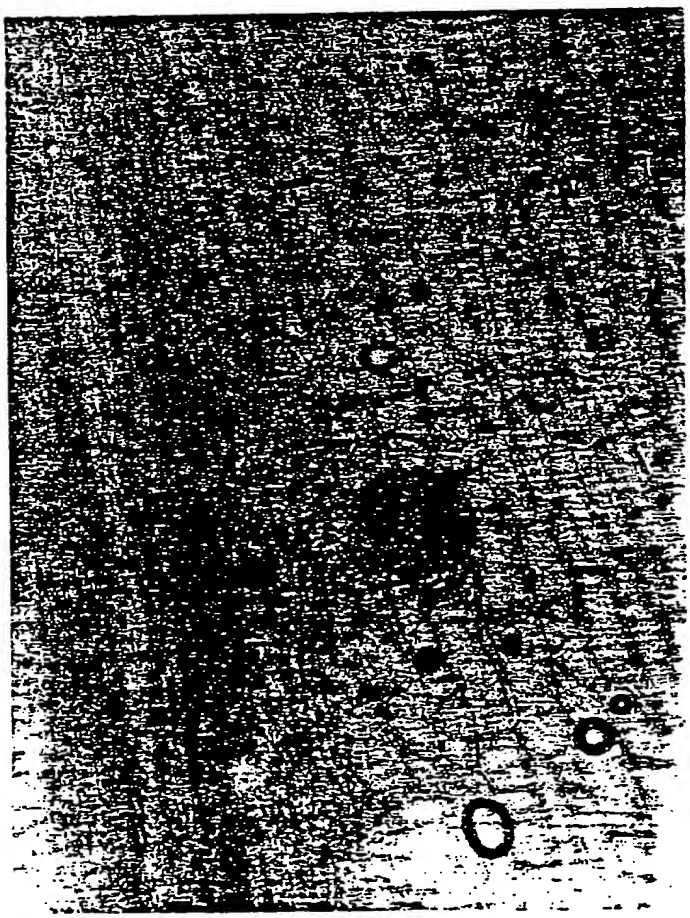

FIG. 19B

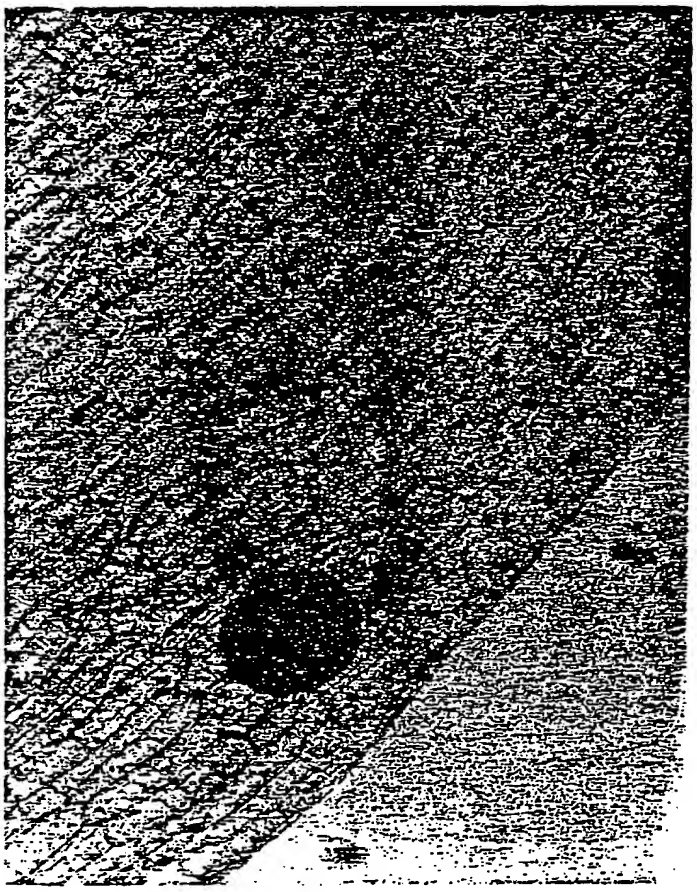

FIG. 19C

FIG. 19D

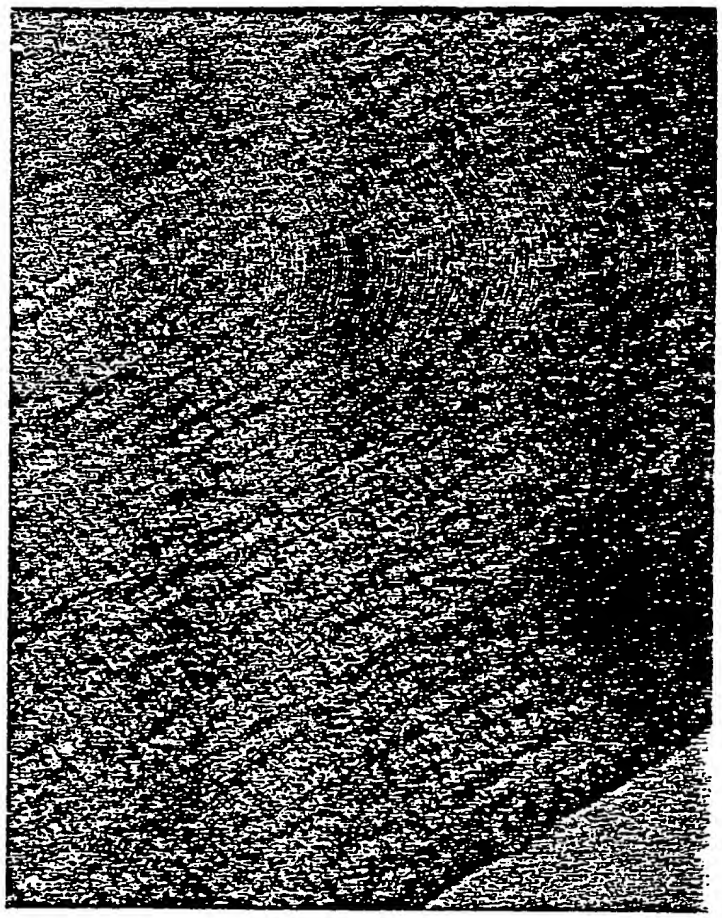

FIG. 20A

FIG. 20B

FIG. 20C

FIG. 20D

FIG. 21A

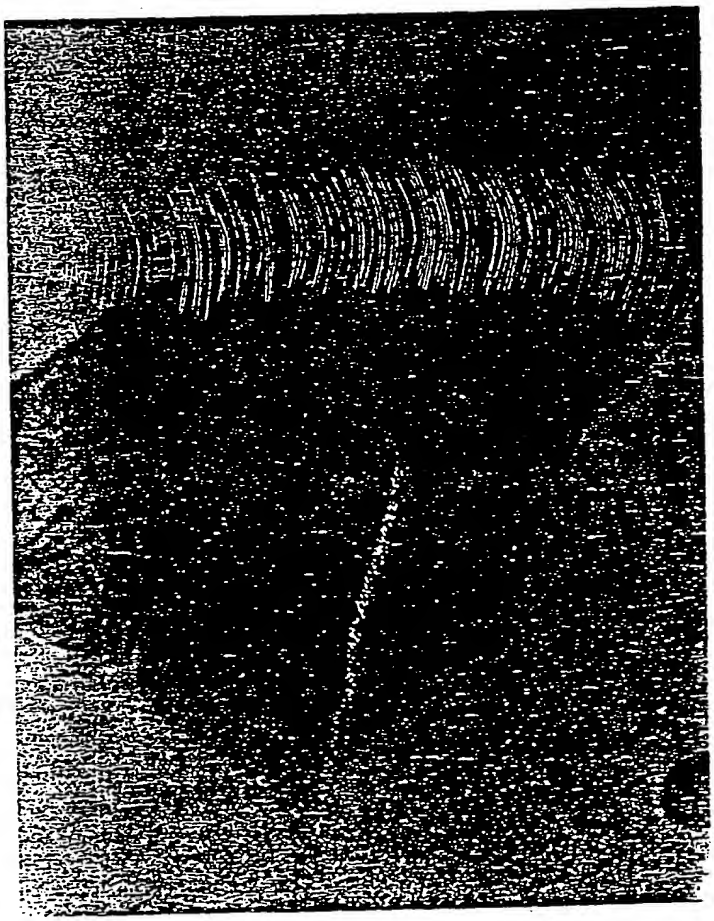

FIG. 21B

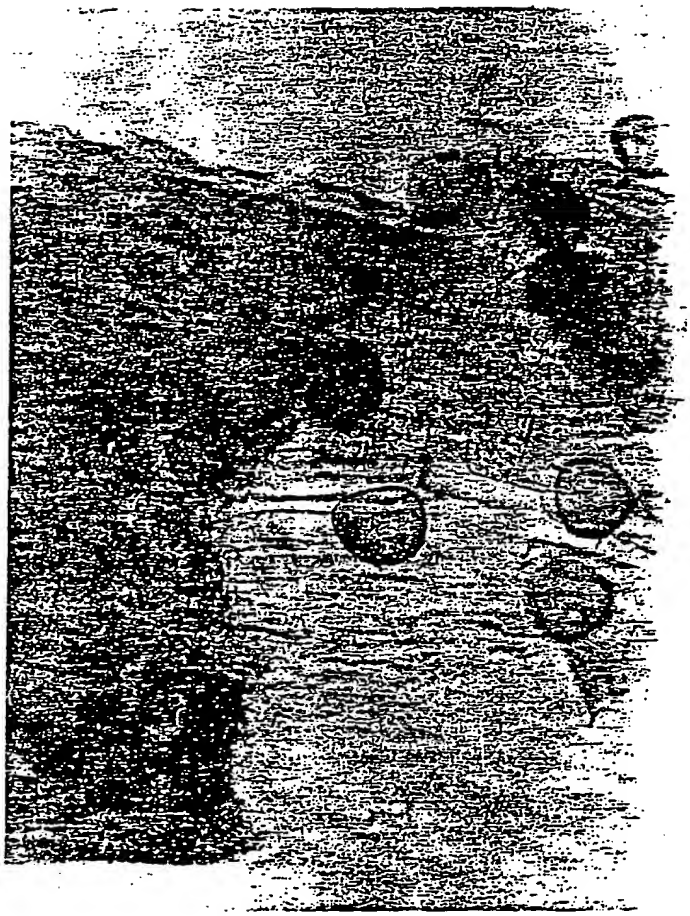

FIG. 21C

FIG. 21D

FIG. 22A

FIG. 22B

FIG. 22C

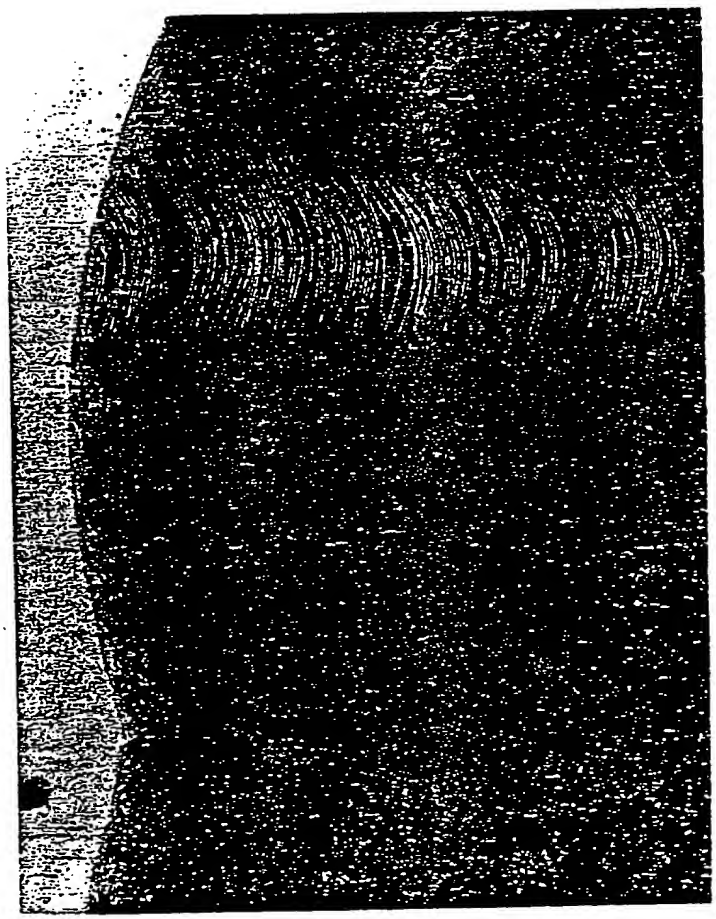

FIG. 22D

I. CLASSIFICATI N OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4):AOIH 1/00;AOIN 65/00;C12N 15/00;C07H 15/12;AOIH 1/04
U.S.C1:47/58;424/195.1;435/172.3;435/320;536/27;800/1

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System [5]	Classification Symbols
U.S.	536/27; 435/172.3; 47/58

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [6]

Databases:DIALOG,File AGRI;Automated Patent System, File USPAT, 1975-1987.
SEE ATTACHMENT FOR SEARCH TERMS.

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	Holl et al., "Genetic Tranaformation in Plants" in H.E. Street, ed., Tissue Culture and Plant Science 1974, published 1974, by Academic Press (London and New York), see pages 301-327.	1-22 and 48-65
Y	Chemical Abstracts, Volume 95, no. 13, issued 1981, September 28 (Columbus, Ohio, USA), R. Scalla et al., "RNA containing intracellular particles in cytoplasmic male sterile faba-bean (Vicia faba L.)" see page 382, column 1, the abstract no. 111926n. Plant Sci. Lett. 1981, 22(3), 269-77 (Eng).	1-22 and 48-65

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [1]	Date of Mailing of this International Search Report [2]
13 October 1988	09 DEC 1988
International Searching Authority [3]	**Signature of Authorized Officer [20]**
ISA/US	Charles E. Cohen

ATTACHMENT TO FORM PCT/ISA/210
Part II. Field Search

Search Terms:

1. Male sterility
2. vector
3. factor
4. DNA
5. RNA
6. nucleic acid
7. transfer
8. transmit
9. transform
10. induce
11. inventors' names

III. DOCUMENTS CONSIDERED T BE RELEVANT (CONTINUED FR M THE SECOND SHEET)		
Category *	Citation of Document, 16 with indication, where appropriate. of the relevant passages 17	Relevant to Claim No 18
Y	Chemical Abstracts volume 95, no. 23, issued 1981. December 7 (Columbus, Ohio, USA), G. Duc et al., "Study of nucleo-cytoplasmic male sterility in the faba bean (Vicia faba L.): presence of cytoplamic particles containing RNA," see page 388, column 1, the abstract no. 200699h, C. R. Seances Acad. Sci, Ser. 3, 1981, 292(23), 1227-30. (Fr.)	1-22 and 48-65
Y	FABIS Newsletter, issued June 1983 (no. 6), Lefebvre et al., "Cyto-plasmic particles associated with male sterility in faba bean (Vicia faba)," page 10.	1-22 and 48-65

ATTACHMENT TO FORM PCT/ISA/210
Observation Where Unity Of Invention is Lacking:

Group I: Claims 1-22 and 48-65, drawn to an AMS/vector and plant extract, and method for asexually inducing male sterility in recipient plants; Class 536/27, Class 424/195.1, and Class 435/172.3.

Group II: Claims 23-47, drawn to male sterile plants, their progeny, and seed; Class 800/1.

Group III: Claims 66-100, drawn to a method for making an F_1 hybrid, and hybrid plants and seeds; Class 47/58 and Class 800/1.

Group IV: Claims 101-136, drawn to a method for inducing apomixis in plants, a method of making apomictic hybrids, and hybird seed; Class 435/172.3, Class 47/58, and Class 800/1.

Group V: Claims 137-141, drawn to a method of delivering a bioacitve molecule and a method of expressing a heterologous gene, and a plant delivery system and a plant expression vector; Class 435/172.3, Class 536/27, and Class 435/320.